CRANFIELD UNIVERSITY

Robert Michael William Ferguson B.Sc.(Hons), M.Sc.

FEEDSTOCKS INFLUENCE ON THE PROCESS PARAMETERS AND THE MICROBIAL COMMUNITY IN ANAEROBIC DIGESTION

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Ph.D. THESIS

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Supervisors: Dr Frédéric Coulon and Dr Raffaella Villa

September 2013

This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Abstract

To improve our understanding into the key parameters controlling and regulating the microbial groups involved in the anaerobic digestion (AD) process, particularly over multiple changes in operational conditions, triplicate lab-scale digesters fed with sewage sludge were exposed to single and multiple changes in organic loading rate (OLR) using either glycerol waste (a by-product of biodiesel manufacture), or Fats oils and greace (FOG waste) collected from a restaurant grease trap. For the multiple changes in OLR, digesters were either exposed to repeated addition of glycerol waste or repeated addition of both glycerol waste and FOG waste. In all conditions tested, physicochemical variables including volatile fatty acids (VFA), alkalinity, pH, biogas production and composition were analysed. Molecular fingerprint techniques including lipid and ether lipid analysis and 454-pyrosequencing of 16S rRNA genes were used to characterise the microbial communities. These techniques were chosen as they complement each other providing information on the microbial biomass and in-depth phylogenetic analysis of the microbial community, respectively. The key question addressed here was how feedstock composition and variation in OLR would affect the microbial community structure and dynamics and relate this to the performance of the digesters in terms of methane production over a long-term period (> 120 days). Multiple changes in OLR with the same feedstock resulted in faster recovery of methane production (8-10 days faster) compared to digesters exposed to single changes in OLR. This finding was associated specifically with a higher proportion of *Clostridia Incertae Sedis* XV (closely related to *Cloacibacillus* genus (83% similarity), *family Synergistaceae*) in the pre-exposed digesters. It is speculated that members related to *Clostridia Incertae Sedis* XV play an important role in the syntrophic interactions with the methanogens. Analysis of the VFA profiles supported this by showing that the higher relative abundance of *Cloacibacillus* was related to higher acetic acid concentrations. The pyrosequencing analysis further showed that community evenness was correlated with the best biogas methane content and shifts in specific bacterial groups was clearly correlated with digester performance. Overall the findings of this PhD provide new insights into the relationships between microbial community structure and digester performance. It also provides new-evidence based knowledge on how molecular microbiological tools can be used in the future to optimise and manage AD plants.

Acknowledgments

Firstly I would like to express my gratitude to my supervisors Dr Frédéric Coulon and Dr Raffaella Villa both of whom have been extremely generous with their time during my PhD and have given valuable advice on all aspects of this project.

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Most importantly I would also like to thank my wife Sophie Ferguson for her love and support. I hope that I can be as supportive during the final months of your PhD. I promise that I will finish digging up the hedge now that the excuse of "I got to write up my PhD" is no longer valid.

Finally I would like to thank my cat Barabas for his casual indifference to my PhD.

And last, but not least, thank you the social club sandwich team for feeding me for the last 3 years.

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Abbreviations

AD	Anaerobic digestion
ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
BMP	biomethane potential
C/N	Carbon Nitrogen ratio
CloVR	Cloud Virtual Resource
DNA	Deoxyribonucleic acid
EPSRC	Engineering and Physical Sciences Research Council
FAME	Fatty acid methyl esters
GC	Gas chromatography
GC -FID	Gas chromatograph with flame ionization detector
GC-MS	Gas chromatograph with mass spectrometry
HPLC	High-performance liquid chromatography
HRT	Hydraulic retention time
ISTD	Internal standard
LCFA	Long chain fatty acid
nMDS	non-metric multi dimensional scaling
MSW	Municipal solid wastes
NCBI	National Centre for Biotechnology Information
OLR	Organic loading rate
OTU	Operational taxonomic unit
P:A	Ration of propionic to acetic acid
PCR	Polymerase Chain Reaction

pH	“power of hydrogen” negative log of hydrogen ion concentration
PLEL	Phospholipids ether lipids
PLFA	Phospholipids fatty acids
Qiime	Quantitative Insights Into Microbial Ecology
RDP	Ribosome Database Project
rRNA	Ribosomal ribonucleic acid
TOC	Total organic carbon
TS	Total solids
VFA	Volatile Fatty acids
VS	Volatile solids
V/A ratio	Ratio of VFA to Alkalinity

Introduction

AD is a key renewable energy technology and has been evaluated as one of the most efficient and environmentally beneficial technologies for conversion of biomass into energy (Chynoweth *et al.* 2001; Panwar *et al.* 2011; Weiland 2011). In addition to renewable energy production AD can be used to close the loop between production and utilisation of organic wastes by optimal recycling rather than landfilling which results in greenhouse gas emissions and leaching of nutrients into the environment (Holm-Nielsen *et al.* 2009). Biogas production from AD has a number of advantages over other renewable energy technologies, it does not require new infrastructure for distribution of energy as the biogas can use the existing national gas grid, and in contrast to solar and wind energy, the biogas can be easily stored and produced when needed to meet demand. It is estimated that AD may be able to produce around 25% of bioenergy required to meet the EU renewable energy policy, which sets a goal of supplying 20% of the European energy from renewables by year 2020 (Holm-Nielsen *et al.* 2009). AD also helps to meet the requirement to divert organic wastes from landfills which is required by the European Landfill Directive (Murphy and McKeogh 2004; Murphy and Power 2006; Ward *et al.* 2008). To meet these ambitious goals, process optimisation of AD is required as digesters often experience process instability, which is mainly due to a limited understanding of the microbial dynamics of the process (Appels *et al.* 2008; Ward *et al.* 2008; Werner *et al.* 2012).

In the past decade the need to divert wastes from landfill, the requirement for the generation of renewable energy, and the requirement to reduce green house gas emissions has lead to the application of anaerobic process to a wide variety of new wastes, including

food wastes, municipal solid wastes (MSW), distillery wastes, lipid rich wastes, industry wastes, farm wastes, and slaughter house wastes (Mata-Alvarez *et al.* 2000; Bouallagui *et al.* 2005; Xiaohua and Jingfei 2005; Clemens *et al.* 2006; McMeekin 2007; Ward *et al.* 2008; Alves *et al.* 2009; Bishop and Shumway 2009; Kryvoruchko *et al.* 2009; Siles *et al.* 2010; Weiland 2011; Iacovidou *et al.* 2012; Ashekuzzaman and Poulsen 2011). The microbial ecology of the process is not well understood, changes in the community can occur without detectable changes in gas production and reactor performance, and *vice versa* (Fernández *et al.* 1999; Zumstein *et al.* 2000; Wang *et al.* 2010b; Wang *et al.* 2011). This has lead to a perception that the technology is inherently unstable. Most of these problems occur as a result of inadequate operational and process control and a lack of understanding of the dynamics of the microbial processes taking place in the digesters (Leitao *et al.* 2006). To increase uptake of the technology an improved understanding of the microbial ecology is required in order optimise the technology and improve investor confidence.

AD of organic wastes includes four main steps hydrolysis, acidogenesis, acetogenesis and methanogenesis, the first three steps are completed by bacteria and the final one by archaea (Braber 1995; O'Flaherty *et al.* 2006). The microbial ecology of AD is a highly dynamic process with many complex ecological interactions including competition, mutualism, antagonism and syntrophism affecting the function of digesters. There is consensus among the scientific community that in-depth understanding of the microbial ecology of AD is vital to optimise and manage the AD process (Briones and Raskin 2003; Rittmann 2006; Rittmann *et al.* 2006; Werner *et al.* 2012). Despite this, microbial ecology is often treated as a 'black box' by plant operators and optimisation and

management of AD is based mainly on the physicochemical parameters of AD. The proliferation of new nucleic acid based techniques to characterise microbial communities without the need for culturing has allowed microbial ecology to advance rapidly during the past 20 years. This has led to a number of studies analysing the microbial communities in AD, both at laboratory scale (Delbès *et al.* 2000; Kaewpipat and GradyJr 2002; Sundh *et al.* 2003) and on full-scale reactors (LaPara *et al.* 2000; Angenent *et al.* 2002; McMahon *et al.* 2004; Karakashev *et al.* 2005; Keyser *et al.* 2006). Despite the amount of literature available, it is difficult to compare and collate the data due to the diversity of conditions and methods used, additionally unstable communities have been observed in digesters with stable performance, as a result it has been hard to understand the relationship between microbial community response and digester performance (Fernández *et al.* 1999; Zumstein *et al.* 2000; Wang *et al.* 2010b; Wang *et al.* 2011). Many trials are short term and do not consider the long-term development of the microbial community. It has been shown that over the long-term operation of AD the performance can deteriorate due to VFA accumulation, studies have also shown that the history of the microbial community can affect subsequent performance (Briones and Raskin 2003; McMahon *et al.* 2004; Palatsi *et al.* 2009; Palatsi *et al.* 2010; Banks *et al.* 2012). It is important to further develop our understanding of the long term development of microbial communities in AD as short term trials and biomethane potential tests (BMP) may not provide a comprehensive analysis of how feedstocks will perform in AD in a realistic operational setting.

Key knowledge gaps are how the microbial community responds to multiple changes in conditions over long-term operation of AD. In addition to this the dynamics of the bacterial community are less well understood in comparison with the archaeal

community. The research presented in this thesis provide insights into both of these problems. The effects of multiple changes in feedstock composition and OLR on the AD performance and microbial communities were assessed using co-digestion of two feedstocks mixed with sewage sludge over long term trials. To develop understanding of the bacterial community a combination of lipid fingerprinting and 454-pyrosequencing was used to provide a unique insight into the structure and abundance of bacterial groups in AD.

Aims and Objectives

This PhD research aimed at improving methane production and process stability in AD by gaining a better understanding of the microbial ecology that drives the process. The influence of operational parameters, such changes in organic loading rate (OLR) and feedstock types on the microbial community response was investigated to identify the key microbial groups involved and determine the relationships between microbial community dynamics and digester performance.

Specifically, the objectives of this PhD study were as follows:

1. To critically review the microbial ecology and diversity within AD studies and shed light the value and potential of using molecular microbiology based techniques to optimise AD process. (Chapter 1)

2. To evaluate the effects of changes in OLR and feedstock composition on the AD process in terms of volatile fatty acid production, biogas production and AD performance in terms of methane production. (Chapter 2 and 4)

3. To characterise microbial community diversity, biomass and dynamics in response to feedstocks and OLR change (Chapter 3 & 4)
4. To identify the key members involved in the biological stability of anaerobic digesters using in depth mining 454-pyrosequencing analysis (Chapter 5) and
5. To establish the relationships between the key physicochemical parameters and the microbial community changes in order to optimise AD process (Chapter 5)

Project Development

The work presented in this document was funded by the Questor Centre and the Engineering and Physical Sciences Research Council (EPSRC) to improve methane production and process stability in AD through enhancing our understanding of the microbial ecology of the technology (Questor project CRA1/09/12).

Thesis structure

The experimental work was carried out by Robert Ferguson at Cranfield University with the exception of the DNA extraction and 454-Pyrosequensing which was carried out at the Food and Environment Research Agency (FERA). All chapters were written by the primary author and edited by Dr Frédéric Coulon and Dr Raffaella Villa (supervisors). This thesis is presented as a series of chapters (Table 1).

Table 1. Summary of thesis plan.

Chapter	Title	Objective
1	Bioengineering options and strategies for optimising anaerobic digestion process.	1
2	The effect of feedstock and OLR change on AD performance during co-digestion.	2
3	The effect of feedstock and OLR changes on microbial community during co-digestion.	3 & 4
4	The effect of feedstock and OLR change on volatile fatty acids (VFA) production and microbial community dynamics in anaerobic digestion.	2, 3, & 4
5	Microbial community dynamics and anaerobic digester performance	4 & 5
6	Key findings and Implications	

Chapter 1: Literature Review. Bioengineering options and strategies for optimising anaerobic digestion process

A literature review was carried out to summarise the current knowledge of how key physiochemical parameters such as feedstock, organic loading rate, volatile fatty acids, ammonia, and trace metals influence the microbial community in AD. Insights into how this information could be used for process optimisation were further discussed.

Chapter 2: The effect of feedstock and OLR change on AD performance during co-digestion

Digesters were exposed to single and multiple changes in OLR with either single feedstock or multiple feedstocks. The novelty of this is the use of multiple stress events

and also multiple substrates. Previous studies have used either single stress events (Peck *et al.* 1986; Hashsham *et al.* 2000; Delbès *et al.* 2001; Sundh *et al.* 2003; Wu *et al.* 2006; Gomez *et al.* 2011; Chen *et al.* 2012; Lerm *et al.* 2012) or multiple stress events with one feedstock (Xing *et al.* 1997; McMahon *et al.* 2004; Palatsi *et al.* 2009; Palatsi *et al.* 2010). The aim of this chapter was to understand if the performance of the digesters was affected by multiple changes in conditions. The work also evaluated which monitoring parameters consistently predicted performance in the digesters and if the values/relationship was affected by multiple stress events. The research provides valuable information to AD operators on the long-term implications of inconsistent OLR and feedstock composition on AD.

Chapter 3: The effect of feedstock and OLR changes on microbial community during co-digestion

A combined approach of lipid fingerprinting (PLFA and PLEL) and 454-pyrosequencing was used to determine how the microbial community structure and biomass was affected by multiple changes in OLR and feedstock. The novelty of this part of the research is the unprecedented details on both the phylogenetic structure and biomass of the microbial community present in AD. Previous studies have either employed lipid fingerprinting alone (Sundh *et al.* 2003; Schwarzenauer and Illmer 2012) or 454- pyrosequencing in combination with other PCR based methods (Schlüter *et al.* 2008; Kröber *et al.* 2009; Zhang *et al.* 2009; Lee *et al.* 2012). The integrated approach taken in this work provides a valuable and comprehensive insight into the abundance, the diversity, and the structure of the community in response to feedstock changes.

Chapter 4: The effect of feedstock and OLR change on volatile fatty acids (VFA) production and microbial community dynamics in anaerobic digestion.

The production and composition of VFA after OLR and/or feedstock type changes was investigated to understand whether different feedstocks were processed in a different way by the microbial community and if this would affect the performance and recovery of the digesters as shown in previous research (Hashsham *et al.* 2000). The relationship between microbial community and VFAs was also investigated. Although the relationship between archaeal community and VFA is well understood (Griffin *et al.* 1998; Delbès *et al.* 2001; Hori *et al.* 2006; Wang *et al.* 2009b; Ros *et al.* 2013) the influence of VFA on the bacterial component of the community is less certain with inconsistency in the research literature. The use of a combined approach of lipid fingerprinting and 454- pyrosequencing has provided new insights into the effect of VFA on bacterial community structure.

Chapter 5: Relationship between microbial community dynamics and anaerobic digester performance

Previous research has suggested that the microbial diversity and the functional redundancy present in AD systems may prevent a consistent and clear relationship between microbial community structure and function (Fernández *et al.* 1999; Feng *et al.* 2011). The aim of Chapter 5 was to further investigate whether a relationship between the microbial community structure and the digester performance can be established and

to demonstrate how molecular based techniques can be used to optimise and manage AD plants.

Chapter 6: Key findings and Implications.

The key findings of the research undertaken and the wider implications were summarised in chapter 6. Recommendations for AD operators are made as well as further research topics.

Chapter 1: Bioengineering options and strategies for the optimisation of anaerobic digestion processes

Abstract: Anaerobic digestion (AD) is a complex biological process and the microbial diversity and dynamics within the reactor needs to be understood and considered when process optimisation is sought after. Microbial interactions such as competition, mutualism, antagonism and syntrophism affect the function and the survival of single species in the community; hence they need to be understood for process improvement. Although the relationship between process performance and microbial community structure is well established, changes in the community might occur without detectable changes in gas production and reactor performance. Recent molecular based studies have highlighted the complexity of AD systems revealing the presence of several uncultivated species and the need for further research in this area. However, this information is still rarely used for process optimisation. The integration of next generation sequencing technologies, such as 454-pyrosequencing, with other techniques, such as phospholipids-derived fatty acids analysis can provide an holistic understanding of the microbial community. In addition, the in depth phylogenetic resolution provided can aid environmental ecologists and engineers to better understand and optimise AD process and consolidate the information collected to date.

1.1. Introduction

Anaerobic Digestion (AD) is the biological conversion, in the absence of oxygen, of organic waste into biogas (comprising methane and carbon dioxide). The AD process is an attractive waste management strategy as it has a number of useful outputs, including biogas, heat and digestate (Parkin and Owen 1986; Sekiguchi *et al.* 2001; Chan *et al.* 2009; Weiland 2011). The use of the anaerobic process to treat wastewater sludge solids and high-strength organic wastes is well established. However, in the past decade the need to divert wastes from landfill, the requirement for the generation of renewable energy, and the requirement to reduce greenhouse gas emissions has lead to the application of anaerobic process to a wide variety of new wastes, including food wastes, municipal solid wastes (MSW), distillery wastes, farm wastes, and slaughter house wastes (Mata-Alvarez *et al.* 2000; Bouallagui *et al.* 2005; Clemens *et al.* 2006; Bishop and Shumway 2009; Kryvoruchko *et al.* 2009; Weiland 2011; Iacovidou *et al.* 2012). Although AD is an established technology the process is often run well below its full potential and optimisation of this technology is still required, particularly in the context of digesting new feedstock types (Mata-Alvarez *et al.* 2000; Ward *et al.* 2008).

The optimisation of the AD process has mainly been focused on the operational parameters such as reactor configuration, mixing, temperature, feedstock composition and pre-treatment of wastes (Stafford 1982; Ince 1998; Angelidaki *et al.* 2000; Mata-Alvarez *et al.* 2000; Stroot *et al.* 2001; Demirer and Chen 2005; Khanal *et al.* 2007; Appels *et al.* 2008; Ward *et al.* 2008; Apul and Sanin 2010; Bouallagui *et al.* 2010; Wang *et al.* 2010a). For example, co-digestion of different waste material, which has a number of potential benefits in AD including improving the overall availability of nutrients and the dilution of inhibitory compounds, has been effective in improving AD

of new waste streams (Callaghan *et al.* 1999; Chen *et al.* 2010a; Chen *et al.* 2010b; Siles *et al.* 2010; Zhang *et al.* 2010; Zhang and Banks 2012; Zhang *et al.* 2012b). Co-digestion of algal sludge with waste paper and co-digestion of cattle slurry with vegetable wastes and chicken manure have both been shown to result in a doubling of methane yields (Callaghan *et al.* 2002; Yen and Brune 2007). However, co-digestion has also been shown to cause changes in the microbial dynamics in AD (Wang *et al.* 2009; Martin-Gonzalez *et al.* 2011; Supaphol *et al.* 2011). Although tools have been developed to optimise co-digestion based on AD operational performance and parameters, this has still to be done to identify and optimise the microbial communities involved in the process (Ãlvarez *et al.* 2010).

AD is a biological process therefore it is also important to understand the microbial diversity and dynamics within the digesters. It is well known that factors such as mixing, feedstock composition, and OLR/HRT can influence the structure and dynamics of the microbial community in AD (McMahon *et al.* 2001; Akarsubasi *et al.* 2005; Kaparaju *et al.* 2008; Rincón *et al.* 2008; Wang *et al.* 2009a; Gomez *et al.* 2011; Martin-Gonzalez *et al.* 2011; Supaphol *et al.* 2011; Bialek *et al.* 2012; Chen *et al.* 2012). In contrast, it is less well known how the structure of the microbial community influences AD performance as changes in microbial community structure can occur without detectable changes in gas production and reactor performance (Fernández *et al.* 1999; Fernández *et al.* 2000; Zumstein *et al.* 2000; Kaewpipat and GradyJr 2002; Wang *et al.* 2010b; Wang *et al.* 2011). It has been suggested that high functional redundancy and microbial population variation between digesters, particularly in the bacterial populations, negates any clear and/or repeatable trend between performance and bacterial community structure (Fernández *et al.* 1999; Kaewpipat and GradyJr 2002;

Feng *et al.* 2011) However, clear relationships between the less diverse archaeal populations and the community structure have been suggested, with *Methanoseta* considered as an indicator of good system health while a shift to *Methanosarcina* could indicate periods of instability in terms of methane production (Demirel and Scherer 2008; Rincón *et al.* 2008; Blume *et al.* 2010). It is possible that the lower diversity of the archaeal community, in comparison to the bacterial community, eases the understanding of the relationships between performance and community structure. However a more detailed analysis of the bacterial community can further help to understand the relationships between AD performance and bacterial community structure.

Advances in culture independent microbiology over the last 20 years, and in particular next generation sequencing (NGS) allow to examine AD microbial communities in far greater depth than previously possible (Schlüter *et al.* 2008; Talbot *et al.* 2008; Kröber *et al.* 2009; Werner *et al.* 2012). This represents an opportunity to develop a deeper understanding of the relationships between AD performance and microbial community structure and function. This review seeks to highlight the value and potential of applying knowledge on the microbial communities involved in AD to achieve process optimisation.

1.2. Overview of the microbial ecology in AD processes

The anaerobic digestion process includes three main conversion steps carried out by the Bacteria, hydrolysis, acidogenesis, and acetogenesis, and one conversion step, methanogenesis, carried out by the Archaea (Braber 1995; O'Flaherty *et al.* 2006). Disturbances at one stage have downstream effects on the other populations that often cause an imbalance in the process. This can result in the accumulation of intermediate

products, indicating that the microbial community is under stress. An imbalance of the conversion products between the acid forming stages and the methanogen stage can cause an increase of volatile fatty acids (VFA) and a drop in pH (Hori *et al.* 2006). Most of the methane-forming Archaea are active at pH values between 6.8 and 7.2 (Garcia *et al.* 2000; Demirel and Scherer 2008). If pH values in the reactor drop below this range, the archaea will be outcompeted by the fermentative bacteria which will continue to produce volatile fatty acids further lowing the pH. In this condition, acetic acid is metabolised through other pathways such as hydrogen production or sulphate reduction and therefore low methane production is reported in digesters (Van Den Berg *et al.* 1980; Conrad 1999; Schnürer *et al.* 1999; Zhang *et al.* 2007; Qu *et al.* 2009; Wang *et al.* 2009b; Laukenmann *et al.* 2011). A theoretical representation of how a microbial community within AD may respond to perturbation is shown in Figure 1.1. A community can either demonstrate resistance (remain the same), resilience (change and return to original state) or adaptation and resulting in either unchanged or improved functionality in performance parameters such as methane production. The factors that influence these outcomes are at present unclear but likely to be related to the magnitude and duration as well as the type of the perturbation applied (pH change, chemical inhibition or temperature for example). The outcomes will also be influenced by the initial microbial community. The key features of the microbial community that will play a significant role are yet to be investigated but could include the existence of syntrophic relationships, the functional characteristics of the individual species, overall species diversity in the community and the distribution of diversity across the community (evenness). By gaining a better understanding of the factors that control the outcomes indicated in Figure 1.1, environmental microbiologists, engineers and operators will be

able to better predict AD performance and therefore to optimise and control the process. A consolidation of the current knowledge of the diversity present in AD, its roles, and how the physiochemical parameters affect them is therefore required to develop microbial optimisation of AD.

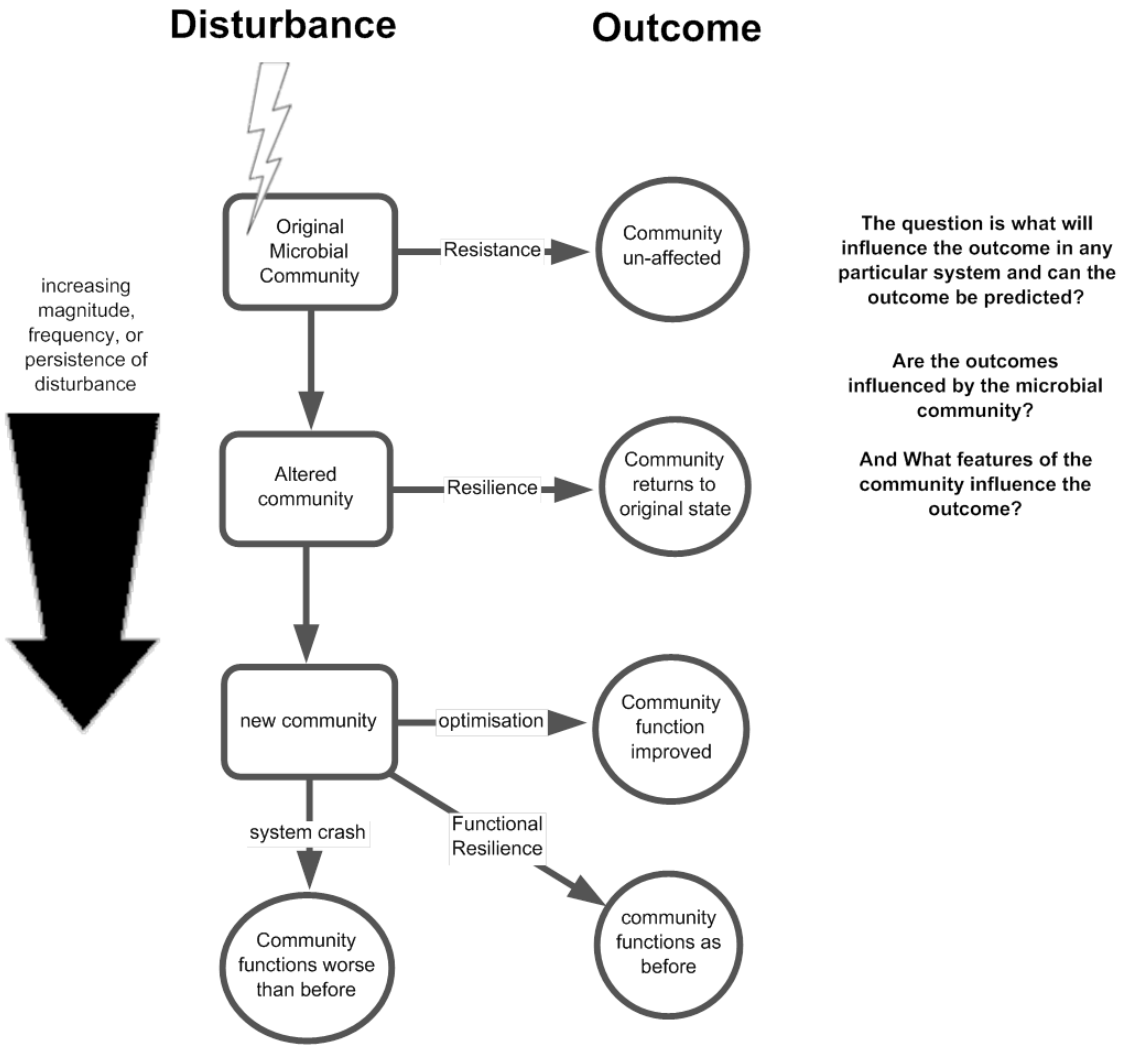


Figure 1.1. Theoretical response of a community to disturbance (adapted from Allison and Martiny, 2008)

1.3. Microbial diversity of anaerobic digestion

1.3.1. Retrieval of sequences from NCBI

To summarise the diversity present in AD a set of sequences was retrieved from the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) using the search term "anaerobic digester". Sequences under 200 bp and those not originating from studies of AD were removed. A total of 3457 bacterial and 2946 archaeal sequences were retrieved. These sequences were aligned and clustered with the Ribosomal Data Project (RDP) pyrosequencing pipeline (http://pyro.cme.msu.edu/) and then classified using the RDP Naive Bayesian rRNA Classifier, (Version 2.5 01/05/12, Taxonomical Hierarchy: RDP 16S rRNA training set 9, Submission Date: 03 Oct 2012) using the default confidence threshold of 80 % to ensure good phylogenetic resolution of all OTUs (Wang *et al.*, 2007). Rarefaction analysis (Figure 1.2) shows that at 5 % phylogenetic distance most of the diversity had been sampled whereas at 10 % phylogenetic distance saturation had been reached.

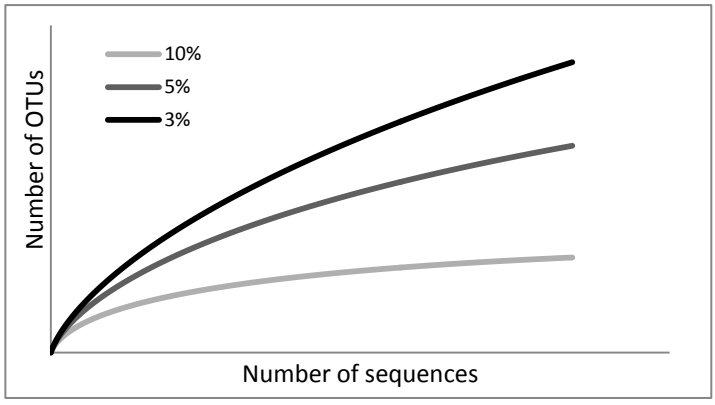


Figure 1.2. Rarefaction curve of OTUs identified using Ribosomal Data Project (RDP) pyrosequencing pipeline per-sequences sampled at 3,5, and 10 % phylogenetic distance.

1.3.2. Bacterial diversity in AD

The bacterial phylum *Firmicutes,* accounted for 1393 sequences. Of these 841 were attributed to the class *Clostridia* and 233 to *Bacilli*. Other phyla included *Proteobacteria* (524 sequences) *Bacteroidetes* (266 sequences) *Chloroflexi* (81 sequences) and *Actinobacteria* (51 sequences) (Figure 1.3). Twenty-five sequences were identified for both *Spirochaetes* and *Synergistets*. Other phyla identified included *Thermotogae, Tenericutes, Lentisphaerae, Armatimonadetes, Acidobacteria, Chlorobi, Deinococcus-Thermus, Planctomycetes, Fusobacteria, Caldiserica, Nitrospira, Verrucomicrobia* and *Fibrobacteres* which all had less than 10 sequences. The ability to classify and list the diversity of the microbial communities in AD has increased with the advent of NGS technologies. However without an understanding of the roles of these groups and how they respond to changes in the physicochemical parameters it remains difficult to optimise AD processes.

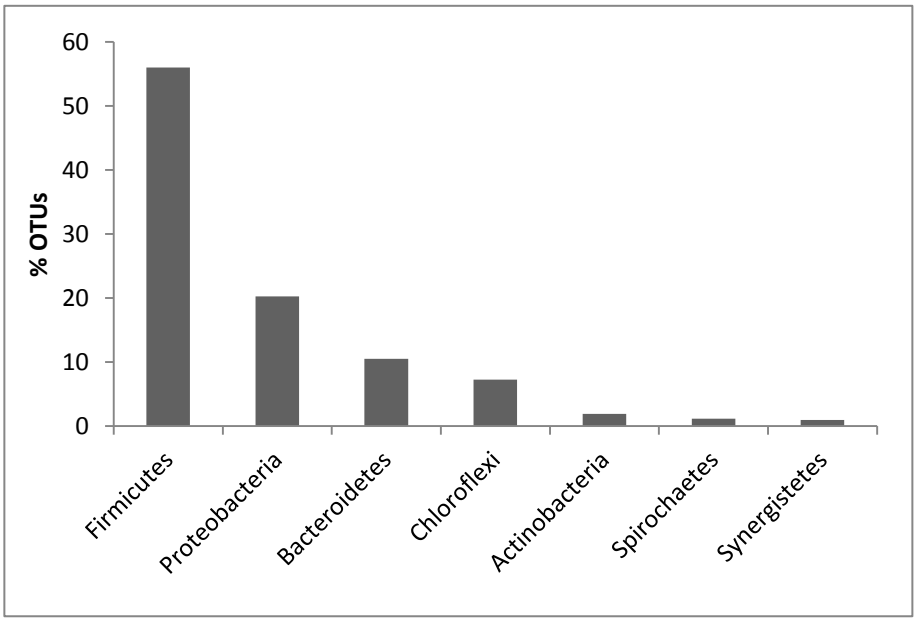


Figure 1.3. Distribution of sequences from anaerobic digesters at Phylum level

1.3.3. The archaeal diversity in AD

Methanogenesis is the final stage of AD and is carried out exclusively by methanogenic archaea belonging to the phylum *Euryarchaeota*. There are five orders of *Euryarchaeota* that can carry out methanogenesis comprising *Methanopyrales, Methanococcales, Methanobacteriales, Methanomicrobiales* and *Methanosarcinales*. All of them are obligate methane producers that derive most or all of their energy from methanogenesis (Garcia *et al.* 2000; Thauer *et al.* 2008). An analysis of the archaeal diversity present in anaerobic digesters based on sequences retrieved from the NCBI Figure 1.4 showed that only the orders *Methanosarcinales* (1514 sequences) *Methanomicrobiales* (504 sequences) and *Methanobacteriales* (246 sequences) are predominant in AD systems. The methanogens feature a limited metabolic diversity with only three main pathways of methane production including the hydrogenotrophic, acetoclastic, and methylotrophic pathways. The hydrogenotrophic pathway is common to almost all methanogens while the acetoclastic and the methylotrophic pathways are restricted to the *Methanosarcinales* (Liu and Whitman 2008; Luo *et al.* 2009). Archaea are less diverse, metabolically slower and less resilient to stress than the bacterial component of the community in AD. Methanogenesis is therefore often considered more susceptible to stress and instability than the other stages (Delbès *et al.* 2001; Leclerc *et al.* 2001; Hori *et al.* 2006; Lee *et al.* 2008; Liu and Whitman 2008). In the following section the effect of parameters such as feedstock and VFA concentration on both the bacterial and archaeal communities are examined.

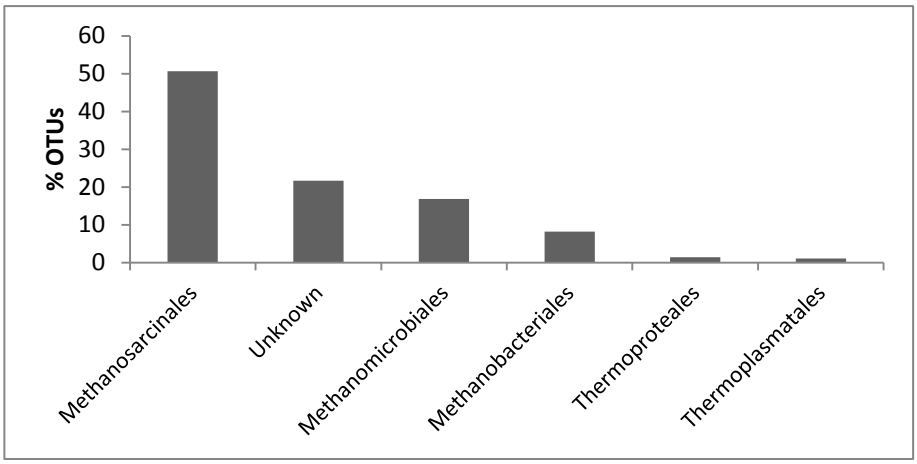


Figure 1.4. Distribution of methanogenic sequences from anaerobic digesters at Order level. 682 sequences were unclassified.

1.4. Influence of physicochemical parameters on microbial communities in AD

1.4.1. Effect of feedstock on microbial community

Bacteria are responsible for the first three stages of AD (hydrolysis, acidogenesis and acetogenesis) and as a result of this, they directly interact with the feedstock composition. This is in contrast with the Archaea which are only able to convert the products of the final bacterial stages into methane. Therefore it would be expected that the structure of the bacterial community, and in particular the hydrolytic bacteria, would be heavily influenced by the feedstock characteristics. Indeed, several studies have shown that feedstock affects the bacterial community structure (Wang *et al.* 2009a; Supaphol *et al.* 2011; Nelson *et al.* 2012; Shen *et al.* 2012; Xia *et al.* 2012). Most of the observed changes are within the hydrolytic groups (Clostridiales and Bacteroidetes Orders). However, the previous generation of culture independent microbiological techniques were biased towards the dominant community members. As mentioned in section 1.3.2 *Clostridia* and *Bacteroidetes* were the most common sequences in AD

studies, it is therefore possible that the results were biased to these bacterial groups and that studies with a higher level of phylogenetic resolution will reveal less dominant but feedstock specific degrading-members. Table 1.1 summarises the dominant bacterial taxa retrieved in NCBI and their possible roles in AD. *Clostridia* are dominant in digesters with high cellulose content (Syutsubo *et al.* 2005; Schlüter *et al.* 2008; Xia *et al.* 2012) whereas *Bacteroidetes* are prevalent in digesters fed with protein rich feedstock such as bovine serum albumin (Tang *et al.* 2005) distillers grains (Ziganshin *et al.* 2011) and casein (Kampmann *et al.* 2012). The *Deltaproteobacteria* and *Actinobacteria* are associated with the digestion of lipid rich wastes and are involved in the beta-oxidation of long chain fatty acids (LCFA) (Cirne *et al.* 2007; Sousa *et al.* 2007; Sousa *et al.* 2008).

The relationship between microbial community and the feedstock is an important factor in AD optimisation, particularly as AD expands to new feedstocks and co-digestion substrates. Changes in feedstock and co-digestion substrate can influence the microbial communities of the digesters and have subsequent consequences on the methane yields and the digesters stability. Such issues need to be understood to ensure optimal AD performance. The core populations needed for the optimal digestion of different feedstocks need to be identified so that AD operators can ensure optimal conditions for AD process.

Table 1.1. Summary of the bacterial Phyla and Classes associated with feedstock type in anaerobic digestion.

Phyla	Class	OTUs	Main Roles	References
Firmicutes	*Clostridia*	1393	hydrolytic (cellulose) acetogenesis	(Xu *et al.* 2003; Syutsubo *et al.* 2005; Schlüter *et al.* 2008; Suen *et al.* 2011)
	Bacilli	233	acetogenesis	(Shin *et al.* 2010)
Proteobacteria	*α-proteobacteria*	23	acetogenesis	(McMahon *et al.* 2004; Ariesyady *et al.* 2007a; Ariesyady *et al.* 2007b; Sousa *et al.* 2008; Werner *et al.* 2012)
	β-proteobacteria	108	acetogenesis	
	δ-proteobacteria,	159	acetogenesis and LCFA oxidation	
	γ-proteobacteria	158	acetogenesis	
	ε-proteobacteria	67	acetogenesis	
Bacteroidetes	*Bacteroidia*	142	protein hydrolysis and amino acid fermentation	(Tang *et al.* 2005; Ziganshin *et al.* 2011; Kampmann *et al.* 2012)
Chloroflexi	*Anaerolineae*	81	syntrophic LCFA oxidation of VFA	(Sekiguchi *et al.* 2000; Sekiguchi *et al.* 2003; Yamada *et al.* 2007; Yamada and Sekiguchi 2009)
Actinobacteria		51	LCFA oxidation and digester foaming	(Rossetti *et al.* 2005; Shen *et al.* 2007; Seviour *et al.* 2008; Ganidi *et al.* 2009; Nielsen *et al.* 2009)
Synergistets		25	amino acid fermentation syntrophic acetogenesis	(Baena *et al.* 1998; Menes and Muxí 2002; Diaz *et al.* 2007; Vartoukian *et al.* 2007)

1.4.2. Effects of organic loading rate (OLR) on the microbial community in AD

OLR is a key parameter for AD operators as higher OLR corresponds to a greater amount of wastes processed. The OLR clearly affects the bacterial community present in AD as increase in OLR has been shown to change the amount and composition of VFA produced by the acidogenic bacteria and therefore influencing the metabolic function of the bacterial community (Wijekoon *et al.* 2011). Rincón *et al.* (2008) showed that increasing the OLR from 0.7 to 9.1 kg VS m^{-3} day^{-1} resulted in a greater bacterial diversity with a shift from a *Clostridium* dominated community to a community comprising members of *Gammaproteobacteria, Actinobacteria, Bacteroidetes* and *Deferribacteres*. Krakat *et al.* (2011) also observed relationships between OLR and bacterial community structure with an increase of *Acidobacteria* and *Chloroflexi* (> 65 % of clones) at the highest OLR (13 VS m^{-3} day^{-1}) and a decrease of *Planctomycetes, Alcaligenaceae.*

In regards to OLR effect on the methanogens, contrasting results have been reported. For example, Rincón *et al.* (2008) reported dominance of *Methanosaeta* at OLR ranging from 0.7–9.1 kg VS m^{-3} day^{-1} and Gomez *et al.* (2011) also reported no change in an archaeal community comprising *Methanomicrobiales, Methanosarcinales and Methanobacteriales* at OLR ranging from 3.4 to 5.0 kg VS m^{-3} day^{-1}. In contrast, Montero *et al.* (2008) observed an increase in *Methanosaeta* (acetotrophic methanogens) from 1 % to 30 % as OLR was increased from 4.4 to 7.2 kg VS m^{-3} day^{-1} and a corresponding decrease of *Methanobacteriaceae* from 11 % to 7 % (hydrogenotrophic methanogens). Further to this, Lerm *et al.* (2012) recently showed a reverse relationship with a switch from *Methanosarcina* to the exclusively hydrogenotrophic methanogens *Methanospirillum* and *Methanoculleus* as OLR was

increased from 2.5 to 40 kg VS m^{-3} day^{-1}. The archaeal shift observed by Lerm *et al.*(2012) can be related to a significant increase in the VFA concentration and/or an organic overload of the digesters suggesting that OLR only affects the archaeal community if it results in changes in other parameters such as VFA concentration an pH.

Overall the studies discussed here on the influence of OLR on the Bacteria and Archaea involved in AD highlighted contrasting effects and therefore the difficulty of developing a predictive understanding of the relationship between digester performance and microbial community structure and dynamics.

1.4.3. Effects of VFA composition and concentration on microbial community

VFA are intermediate products produced during acidogenesis and acetogenesis. Acetic acid is the key substrate for methanogenesis, but if its production rate is faster than its utilisation rate by the methanogens, digester instability will occur. (as discussed in section 1.2.). For example, shift in the archaeal population from *Methanosaeta* to *Methanosarcina* in digesters experiencing a VFA increase from to more than 1.5 g l^{-1} has been observed by several authors (Griffin *et al.* 1998; Karakashev *et al.* 2005; Karakashev *et al.* 2006; Ros *et al.* 2013). Hori *et al* (2006) also showed that VFA accumulation resulted in a 10,000 fold increase in the gene expression for the hydrogenotrophic methanogen *Methanothermobacter* and Delbès *et al.* (2001) reported the dominance of *Methanobacterium* at high acetic acid concentrations (> 3 g/l). Analysis with fluorescence in situ hybridization (FISH) indicated that syntrophic interactions between hydrogenotrophic methanogens and bacteria is key in the degradation of VFA and recovery of digesters (Hori *et al.* 2006). McMahon *et al.*

(2004) further showed that digesters with a history of poor performance had proportionally higher numbers of *Methanosarcina* than *Methanosaeta* as well as a higher number of syntrophic bacteria including *Syntrophobacter, Smithella, and Syntrophomonadaceae,* and consequently that the microbial community of the digesters was more tolerant to VFA accumulation as a result of the high organic loading. These results demonstrate that the structure of the microbial community can positively influence AD performance. Clearly higher numbers of syntrophic Archaea and bacteria are desirable for AD which can be increased by past stress in the reactor. This can be related to Figure 1.1 as a perturbation altering the community structure and resulting in optimised performance.

Although the relationship between the VFA concentration and the archaeal community dynamics are well understood, our understanding of the relationship between the bacterial community dynamics and the VFA composition and concentration is still limited. A relationship between acetic acid concentration and *Clostridia* abundance was proposed by Delbès *et al* (2001). However in a previous study, Delbès *et al.* (2000) reported no difference in the bacterial response to acetate, propionate, or butyrate suggesting that quantifying the total concentration of VFA was more important than reporting the concentration of speciated VFA. This finding is somewhat surprising as bacteria are the VFA producers in AD and therefore likely to influence the VFA composition and concentration in AD systems. Also it has been demonstrated that propionic acid has a higher inhibition effect than acetic and butyric acids on methane production (Demirel *et al.* 2002; Wang *et al.* 2009b). This controversial finding reinforces the need to further investigate the relationship between specific archaeal/bacterial groups and specific VFA.

1.4.4. Effect of ammonia on the microbial community

Many farm wastes including pig slurries, slaughter wastes, cattle and poultry manure have high concentration of ammonia which can be either inhibitory or beneficial to maintain optimal AD process (Ripley *et al.* 1986; Angelidaki and Ahring 1993; Borja *et al.* 1996; Hansen *et al.* 1998; Angenent *et al.* 2002; Buenda *et al.* 2009). Several studies have reported significant increase of hydrogenotrophic methanogens belonging to *Methanosarcinaceae*, and to a less extent to M*ethanomicrobiales* and *Methanobacteriales* in digesters with ammonia concentration of > 3 g/l (Calli *et al.* 2005b; Karakashev *et al.* 2005; Xia *et al.* 2011). The formation of multicellular units by *Methanosarcinaceae* at high concentrations of ammonia protects them and also results in more efficient syntrophic relationships between methanogens and bacteria for interspecies hydrogen transfer. The hydrogenotrophic methanogens are therefore favoured under this condition instead of the acetotrophic methanogens (Calli *et al.* 2005a; Calli *et al.* 2005b). This can be related to Figure 1.1 as an example of a perturbation (increase in ammonia concentration) causing a shift in the microbial community structure and function (switch to hydrogenotrophic methanongenesis) which can preserve AD function.

In contrast, the effect of ammonia on the bacteria is unclear and results suggest that the methanogens are inhibited well before ammonia affects the bacteria (Chen *et al.* 2008). Koster and Lettinga (1988) showed that the production of VFA by bacteria is not significantly affected by ammonia concentration. They also demonstrated that after exposing digesters to ammonia concentration up to 9 g/l for three weeks, ammonia tolerance by the methanogens was improved by 6 times. This finding suggests that the

microbial community of digesters can be easily optimised to produce methane from feedstocks with high ammonia. This can be related to Figure 1.1 as a disturbance (high ammonia) resulting in an optimised community. To date, while the mechanism of this adaption is not fully understood, the predominance of the hydrogenotrophic methanogens and the favoured syntrophic interactions with the bacteria at high ammonia concentration suggest that the acclimation of the community to high VFA concentrations is related to changes in the community structure.

1.4.5. Effect of trace metals on AD microbial communities

The availability of trace metals in AD and effect on performance has been a major topic of research for over 30 years (Hoban and Van Den Berg 1979; Murray and Van Den Berg 1981; Callander and Barford 1983a; Callander and Barford 1983b; Raju *et al.* 1991; Appels *et al.* 2008). Additions of trace metals such as cobalt, molybdenum, iron, nickel, selenium, and sulphate have been shown to improve methane yields (Murray and Van Den Berg 1981; Wilkie *et al.* 1986; Espinosa *et al.* 1995; Jarvis *et al.* 1997; Zandvoort *et al.* 2006; Facchin *et al.* 2013) improve stability (Kim *et al.* 2002; Climenhaga and Banks 2008; Zhang *et al.* 2008; Pobeheim *et al.* 2010; Jiang *et al.* 2012) and optimize long-term AD performance (Banks *et al.* 2012; Zhang and Jahng 2012) largely through reducing accumulation of VFA. It is known that trace metals such as cobalt, nickel, iron, zinc, molybdenum, and tungsten are important for the activity of the enzymes involved in methane production in AD (Takashima *et al.* 1990; Demirel and Scherer 2011). Despite this the effect of trace metal concentration and addition on the structure of the microbial community structure has not been extensively researched.

Fermoso *et al.* (2008) observed decrease in numbers of *Methanosarcina* and associated decrease in performance parameters under cobalt limited conditions in a UASB reactor treating methanol, results also suggested cobalt addition may be a suitable strategy for recovering *Methanosarcina* populations. Banks *et al.* (2012) showed that the dominant methangenic populations in digesters with high ammonia concentration (4-6 g l^{-1} TAN) and varying trace metals concentrations were *Methanoimicrobiales* (which is consistent with results discussed in section 1.4.5.) but indicates that ammonia concentration was a bigger factor than trace metals in structuring the methanogenic community. Feng *et al* (2011) investigated the effect of additions of cobalt alone, a combination of nickel/molybdenum/boron, or a combination of selenium/tungsten on microbial community structure and AD performance. The best methane production was related to high selenium and tungsten concentration with low cobalt. Trace metal concentration did not influence the relative abundance of the most dominant bacterial population (*Actinobacteria*) but two bacterial populations (both related to *Firmicutes*) were positively correlated with the nickel/molybdenum/boron treatment alone and negatively correlated with nickel/molybdenum/boron combined with cobalt. The archaeal populations showed a much greater correlation with the trace metals with a *Methanoculleus* population positively correlated with selenium and tungsten alone but negatively correlated with nickel/molybdenum/boron and selenium and tungsten when they were supplemented together. However another *Methanoculleus* population was negatively correlated with nickel/molybdenum/boron, A *Methanosarcina* population was positively correlated with the nickel/molybdenum/boron treatment. Feng *et al.* (2011) demonstrated that trace metals influence the structure of the bacterial and archaeal populations in AD, however the response of archaeal populations from the

same genus differed, and when nickel/molybdenum/boron was used in combination with cobalt the correlation was reversed. This shows that the relationship between microbial populations in AD and trace metals is complicated and that different combinations of trace metal supplementations can have antagonistic effects. As it is clear that trace metal concentration is a key parameter in optimizing AD more research is required to understand the influence of trace metals on the microbial community to fully exploit this knowledge to optimise AD.

1.5. Microbial optimisation of AD

1.5.1. Bioaugmentation for AD optimisation

Bioaugmentation with a particular species or consortium of species could allow plant operations to change the existing microbial community so that it is optimised to carry out a specific function (Rittmann and Whiteman 1994; Deflaun and Steffan 2002). Bioaugmentation has been used for the remediation of contaminated soils and ground waters and has also been applied extensively to aerobic wastewater treatment (Rittmann and Whiteman 1994; Vogel 1996; Limbergen *et al.* 1998; Deflaun and Steffan 2002; Satoh *et al.* 2003; Gentry *et al.* 2004; El Fantroussi and Agathos 2005; Nancharaiah *et al.* 2008; Schauer-Gimenez *et al.* 2010). Bioaugmentation has been also applied towards the optimisation of a number of aspects of anaerobic digestion including degradation of problematic feedstocks with high cellulose or lipid content, improvement of recovery from perturbation, and faster start-up times (Table 1.2). However bioaugmentation is not always successful and further research is required to develop bioaugmentation as an optimisation strategy in AD (Westerholm *et al.* 2012). The effect of bioaugmentation on

the indigenous community needs to be examined as interactions such as predation and competition may result in negative effects on the community rather than improved performance. Additionally the survival and integration of the exogenous population into the reactor needs to be examined to establish how to maintain the effect of bioaugmentation over long periods. Perhaps the most important question to address is what species/cultures are going to have a beneficial effect on the community. To answer this question, further research identifying novel species with beneficial physiological traits, as reported by Savant *et al.* 2002) and Savant and Ranade (2004) will be useful. Another important approach will be the analysis of microbial communities in digesters under specific conditions, such as recovering from overload, so that an understanding of what type of community consortium will be desirable for a certain situation can be developed.

Table 1.2. Summary of successful bioaugmentation studies in AD

Optimisation	substrate	Microorganism/s	Benefits	Reference
Feedstock	Cattle manure biofibers	Hemicellulose degrading bacterium	+ 30 % CH_4 potential	(Angelidaki *et al.* 2000)
		Caldicellusiruptor and *Dictyoglomus*	+ 10-24 % CH_4 yield	(Nielsen *et al.* 2007)
	poultry litter	*Clostridium cellulolyticum, and thermocellum, Caldicellulosiruptor saccharolyticum*	Up to 15 % increase in CH_4 production	(Costa *et al.* 2012)
	Lipid rich waste	*Syntrophomonas zehnderi*	Improved CH_4 production rate	(Cavaleiro *et al.* 2010)
		Clostridium lundense	Improved CH_4 production rate	(Cirne *et al.* 2006)
Faster recovery	Oxygen exposure	*Methanosaeta*, *Methanoculleus*, and *Methanospirillum*	70-80 days faster recovery	(Schauer-Gimenez *et al.* 2010)
	Organic overload	a propionate-degrading enrichment culture	25 days faster recovery	(Tale *et al.* 2011)
Improve reactor start-up	pharmaceutical effluent	Anaerobic sludge from plant treating antibiotic effluent	faster reactor start-up time	(Saravanane *et al.* 2001)
Improved resilience	Low pH high VFA	acid tolerant *Methanobrevibacter acididurans*	+ 7-12 % CH_4 production	(Savant and Ranade 2004)

1.5.2. Manipulation of process and AD design for microbial optimisation

An alternative to bioaugmentation is to promote microbial community diversity by changing the operational conditions of AD. Research has shown that digesters with greater flexibility in microbial community structure are more resilient to perturbation than more stable communities (Fernández *et al.* 2000; Hashsham *et al.* 2000). Hashsham *et al.* (2000) showed that digesters that were able to process feed through a network of multiple routes in parallel were more stable than those that processed feed through sequential pathways. Therefore promoting functional diversity in the microbial community is one possibility for improving AD stability. This has been recognised and incorporated into the design of AD systems such as baffled digesters or membrane reactors. Functional diversity can also be promoted by using granular substrates (Briones and Raskin 2003). It was suggested by Briones *et al.* (2003) that incorporating changes in operational conditions such as modifying the OLR can also enhance the functional diversity and performance of the digesters. This was also proposed by McMahon *et al* (2004). More recently Palatsi *et al.* (2009) showed that digesters exposed to repeated LCFA pulses had faster recovery times. Therefore there is mounting evidence that the resilience of AD microbial communities can be enhanced through manipulation of the operational conditions which can be subsequently used to optimise AD process.

1.5.3. Microbial community monitoring as decision support tool for AD performance

The lack of reliable sensory equipment and control systems have been reported as one of the major reasons for AD not being operated at optimal conditions (Ward *et al.*

2008). This was stated in the context of monitoring the biochemical process, however as demonstrated in this review the microbial community must be considered in AD optimisation. Talbot *et al* (2008) in a review of nucleic acid based techniques to characterise communities in AD systems point to the development of laboratory-on-chip systems for eventual on-line monitoring of bioreactors. This technology has already been demonstrated for fast characterization the human gut microbiota, where similar microbial consortium are found (Bjerketorp *et al.* 2008). Microarray chips have also been successful in characterising the archaeal community in anaerobic sludge, and therefore it is appropriate to think that an accurate, viable and cheap method for monitoring microbial communities in AD will be available soon (Franke-Whittle *et al.* 2009).

The development of culture independent techniques such as phospholipids analysis (PLFA), denaturing gradient gel electrophoresis (DGGE), single strand conformation polymorphism (SSCP) and others for microbial community fingerprinting has undoubtedly improved our understanding of microbial communities in AD (Delbès *et al.* 2000; Kaewpipat and GradyJr 2002; Sundh *et al.* 2003; Calli *et al.* 2005a; Calli *et al.* 2005b). Fingerprinting techniques, such as DGGE have proved more effective in studying the less diverse archaeal community than the highly diverse bacterial community. This is due to the relative complexity of the bacterial community which is therefore much more challenging to characterise. Community fingerprinting has allowed microbiologists to look at community shifts in relation to changes in the physicochemical parameters, such as VFA profiles, in AD. The application of the so-called next generation sequencing technologies, such as 454-pyrosequencing, represents one of the most exciting areas of development in AD. Combining 454-pyrosequencing

to phylogenetic microarray has provided a cost effective way of obtaining high-resolution data on microbial community structures and function in AD. Schlüter *et al.* (2008) and Kröber *et al.* (2009) analysed the microbial communities in a production scale AD plant fed with maize silage, green rye and liquid manure. The data obtained in these studies far surpasses the depth of information gained in previous studies based on fingerprinting techniques. For example the identification of less dominant members such as *Syntrophobacterales* and *Synergistia* have been shown to have greater correlation with the changes in performance observed (Werner *et al.* 2012). As NGS technologies become cheaper and the strategies for analysing the data get more refined, our understanding of the structure and function of microbial communities, such as those found in AD reactors will improve exponentially. However, these techniques still provide limited quantitative information. To this end, analysis of the lipid content of the community including phospholipids-derived fatty acids (PLFA) for bacteria and phospholipids etherlipid (PLEL) for Archaea can reveal changes in biomass and function of microbial communities(Gattinger *et al.* 2003; Sundh *et al.* 2003; Oravecz *et al.* 2004; Radl *et al.* 2007; Frostegård *et al.* 2011; Schwarzenauer and Illmer 2012). In particular, Schwarzenauer and Illmer (2012) showed that monitoring PLFA could identify changes in the microbial community associated with changes in AD performance. The relative cheapness of lipid fingerprinting and the use of high throughput PLFA techniques, developed by Buyer and Sasser (2012), make it possible to monitor changes in biomass and lipid structure over long time series, at both lab and potentially full scale operational AD plants. Lipid fingerprinting, which are overlooked by many microbiologists outside soil science, can represent a valuable tool in AD optimisation.

1.6. Conclusions and research gaps

The preceding sections of this review have summarised some of the recent developments in microbial ecology in AD. Culture independent analysis of communities has improved our understanding of the process but even so there is no obvious direct application of this information to deliver significant process optimisation in AD. Industry remains sceptical to the benefit that microbial optimisation can provide and this will remain the case until the benefits of microbial optimisation can be empirically proved. There is a perception that the microbial communities are so diverse that it will not be possible to produce a clear understanding of the role of the microbial community. This perception is supported by research showing highly dynamic communities in stable reactors which gives weight to the argument that community shifts are not related to performance (Fernández *et al.* 1999). As reported in this review there is a great deal of work demonstrating that the methanogenic community is influenced by the concentrations of intermediate products such as VFA and other inhibitory compounds such as ammonia. The factors that influence the bacterial community are less understood, but the improved resolution of NGS in combination with other techniques such as lipid fingerprinting may help to improve our understanding of this aspect of the AD community. McMahon *et al.* (2007) called for the integration of microbial ecology and engineering so that novel approaches to manipulating systems can be developed. For example it has been hypothesised that it may be possible to develop more resilient communities by changing operational conditions, rather than letting a community become specialised (Briones and Raskin 2003; McMahon *et al.* 2007). Potential effects that a perturbation may have on a microbial community in AD are illustrated in Figure

1.1. Filling the gaps of knowledge highlighted on this figure will enable optimisation of the AD process and also contribute to the field of microbial ecology in general.

To fill these research gaps a set of aims and objectives for this PhD research have been defined as follows:

Aims:

To improve methane production and process stability in AD by gaining a better understanding of the microbial ecology that drives the process.

Objectives:

1. To critically review the microbial ecology and diversity within AD studies and shed light the value and potential of using molecular microbiology based techniques to optimise AD process. (Chapter 1)
2. To evaluate the effects of changes in OLR and feedstock composition on the AD process in terms of volatile fatty acid production, biogas production and AD performance in terms of methane production. (Chapter 2 and 4)
3. To characterise microbial community diversity, biomass and dynamics in response to feedstocks and OLR change (Chapter 3 & 4)
4. To identify the key members involved in the biological stability of anaerobic digesters using in depth mining 454-pyrosequencing analysis (Chapter 5) and
5. To establish the relationships between the key physicochemical parameters and the microbial community changes in order to optimise AD process (Chapter 5)

Chapter 2: The effect of feedstock and OLR change on AD performance during co-digestion

Abstract: Research into the optimisation of new feedstocks for AD has recently been identified as one of the targets for the widespread implementation of AD by the UK Government. However, information on degradation yields, degradation rates and their influence on reactor performance for many types of feedstock are still missing. Short term trials that only consider one change in feedstock composition or organic loading rate (OLR) may not provide a true picture of how new feedstocks will effect AD performances both at biochemical and at microbial level. To understand these biochemical and microbial changes lab-scale digesters were exposed to series of OLR changes using a sugar-like feedstock (glycerol waste) and/or a lipid feedstock (fat, oil and greases waste, FOGs) in co-digestion with sewage sludge. Successive changes in OLR using the same feedstock (glycerol waste) resulted in significant improvements in performance (biogas production doubled and methane content increased by 7 %) and shorter recovery times (1.5 HRT) times. In contrast successive changes in OLR with varying feedstock composition resulted in a decrease in performance and no change in recovery times. These results demonstrate that consistency in feedstock composition is important in maintaining optimal performances in AD. Analysis of the key process indicators including alkalinity, pH, propionic:acetic ratio and VFA to total alkalinity ratio (V/A) identified the latter as the most responsive parameter to monitor process performances but also showed that reference values for V/A ratio may vary for different digester conditions.

2.1. Introduction

In the past decade the increased importance of the renewable energy obtained from AD - biomethane has promoted considerable interest in the application of this technology to new feedstocks and co-digestion substrates (Mata-Alvarez *et al.* 2000; Bouallagui *et al.* 2004; Clemens *et al.* 2006; Bishop and Shumway 2009; Kryvoruchko *et al.* 2009; Weiland 2011; Zhang and Banks 2012; Zhang *et al.* 2012b; Zhang *et al.* 2012a). In particular, the co-digestion of glycerol waste, a by-product of biodiesel manufacture, and lipid rich wastes such FOGs has shown potential for improving yields of methane in anaerobic digesters (Angelidaki and Ahring 1992; Fernández *et al.* 2005; Yazdani and Gonzalez 2007; Sousa *et al.* 2008; Alves *et al.* 2009; López *et al.* 2009; Palatsi *et al.* 2009; Palatsi *et al.* 2010; Siles *et al.* 2010) . However, high concentrations of glycerol waste and/or FOGs waste can cause process instability through sludge flocculation (resulting in biomass wash out), direct inhibition, VFA overload, and physical fouling of equipment (Fernández *et al.* 2005; Cirne *et al.* 2006; Cirne *et al.* 2007; López *et al.* 2009; Palatsi *et al.* 2009; Lansing *et al.* 2010; Long *et al.* 2011). More generally, process instability can also be linked to sudden changes in feedstock composition and or organic loading rate (OLR) (Sanchez *et al.* 2005; Akunna *et al.* 2007; Rincón *et al.* 2007). As feedstock availability can fluctuate throughout the year it is often difficult for an operator to maintain these two parameters stable (Akunna *et al.* 2007). It is therefore important that the effects of changes in feedstock composition and OLR on AD performance are fully understood so that management strategies to mitigate negative effects are developed (Ward *et al.* 2008).

The effects of changes in OLR on the performance of AD are well documented. Inconsistent or excessive OLR can affect the balance between acetogenesis and methanogenesis by changing the availability of nutrients (Kugelman and Chin 1971; Pavlostathis and Giraldoâ-Gomez 1991). This imbalance can result in process failure by causing a decrease in pH as a result of changes in the ratio of VFA/alkalinity (V/A ratio) (Sanchez *et al.* 2005; Rincón *et al.* 2007; Rincón *et al.* 2008; Lerm *et al.* 2012). The V/A ratio is similar to the Ripley ratio (partial to total alkalinity estimated by titration) which itself has been demonstrated as a key parameter in predicting AD performance with values > 0.25 indicating poor methane production (Ripley *et al.* 1986; Feitkenhauer *et al.* 2002; Lahav and Morgan 2004; Sanchez *et al.* 2005; Rincón *et al.* 2007). Little is known about how V/A ratio is affected by different feedstocks, and indeed, other parameters such as the ratio of propionic to acetic acid (P:A ratio) (with values of > 1.4 indicting of poor digester performance) may also be related to OLR in AD (Hill *et al.* 1987; Marchaim and Krause 1993). In addition to this AD is biological process and is driven by a consortium of bacteria and Archaea which can adapt and acquire resilience based on past conditions. Therefore the response of AD to changes in feedstock and OLR may not always be consistent.

McMahon al. (2004) compared the performance of two digesters after OLR increase to 18.8 kg VS m^3 day^{-1} for a historically stable digester and a digester that experienced an unstable start-up period with accumulation of acetic acid to > 4 g l^{-1} and propionic to > 2 g l^{-1}. The digester that was historically unstable was able to tolerate the high organic load better as indicated by a lower V/A ratio of < 2 in comparison with 17 and lower accumulation of both acetic and propionic acid. The P:A ratio however was not correlated with performance as it was > 1.4 in the better performing digester in

comparison with approximately 1 in the poorly performing digester. These changes in performance were related to variation in the microbial communities present as a result of the different start-up strategies. Digesters with a history of poor performance during start-up developed microbial communities with higher numbers *Methanosarcina spp.* and key syntrophic bacterial groups (*Syntrophobacter wolinii*, *S. pfennigii*, *S. propionica*, and Syntrophomonadaceae) (Stroot *et al.* 2001; McMahon *et al.* 2004). In agreement to this, Palatsi and Laureni *et al.* (2009) showed that digesters exposed to repeated LCFA pulses had faster recovery times (4 times faster) and increased maximum specific methane production rate from 0.04 to 0.16 g COD CH4 g^{-1} VS day^{-1} and the acetate maximum consumption rate from 0.04 to 0.13 g COD acetate g^{-1} VS day^{-1} when exposed to inhibitory levels of LCFA again. It is therefore important to understand AD in the context of multiple changes in operational conditions as the response of the digester may change over multiple changes in OLR and feedstock.

As process instability remains one of the major issues in AD, a better understanding of the relationship between input-feedstock, physico-chemical parameters governing the process and microbial community composition is required to improve digester performances (Chen *et al.* 2008). This work thus aims to understand how the key physicochemical parameters in AD respond to single and multiple series of changes in OLR during co-digestion of a sugar-like feedstock (glycerol waste) and/or a lipid feedstock (fat, oil and greases waste, FOG waste) as co-substrate in sludge digestion. The research also aims, by assessing and comparing the response of key process indicators (alkalinity, pH, propionic:acetic ratio and V/A ratio) of the different settings, to identify the most responsive parameter for monitoring process changes.

2.2. Methods

2.2.1. Operational parameters

Laboratory-scale semi-continuous digesters consisted of 9 x 1-L borosilicate glass bottles with a 700 ml working volume for the co-digestion of glycerol waste; and 6 x 5-L bottles with a 4.5 L working for all other conditions (Table 2.1). A temperature of 38°C was maintained inside the digesters by emersion in a Perspex water bath with temperature regulated by two Grant G120 water heaters (Grant Instruments Ltd. Cambridge. UK). Digesters were seeded with secondary sludge from the AD plant of a local Sewage Treatment Works (Milton Keynes, UK). Digesters were fed with autoclaved primary sludge (one batch) and operated at an organic loading rate of 1.4 kg VS m^{-3} d^{-1} (digesters 1 to 3 and 10 to 12; Table 1) All digesters were fed three times a week to achieve a hydraulic retention time (HRT) of 7 days. If necessary, pH of feed was corrected to 7.2 by chemical addition of NaOH 0.5 M.

Experimental conditions are summarised in Figure 2.1 and Table 2.1, all conditions were tested in triplicate. Digesters were either exposed to one OLR increase series or two OLR increase series. The effect of a single OLR increase series was investigated using either glycerol waste (experiment 1 digesters 1-3) or FOGs waste (experiment 2 digesters 10-12) in co-digestion with primary sludge. Further to this the effects of repeated OLR increase series was investigated using consistent co-digestion substrate (glycerol waste only, experiment 1 digesters 4-6) or inconsistent co-digestion substrate (glycerol then FOG waste, experiment 2 digesters 13-15) in co-digestion with primary sludge. The effect of a higher OLR during the first OLR increase series was also investigated (experiment 1 digesters 7-9).

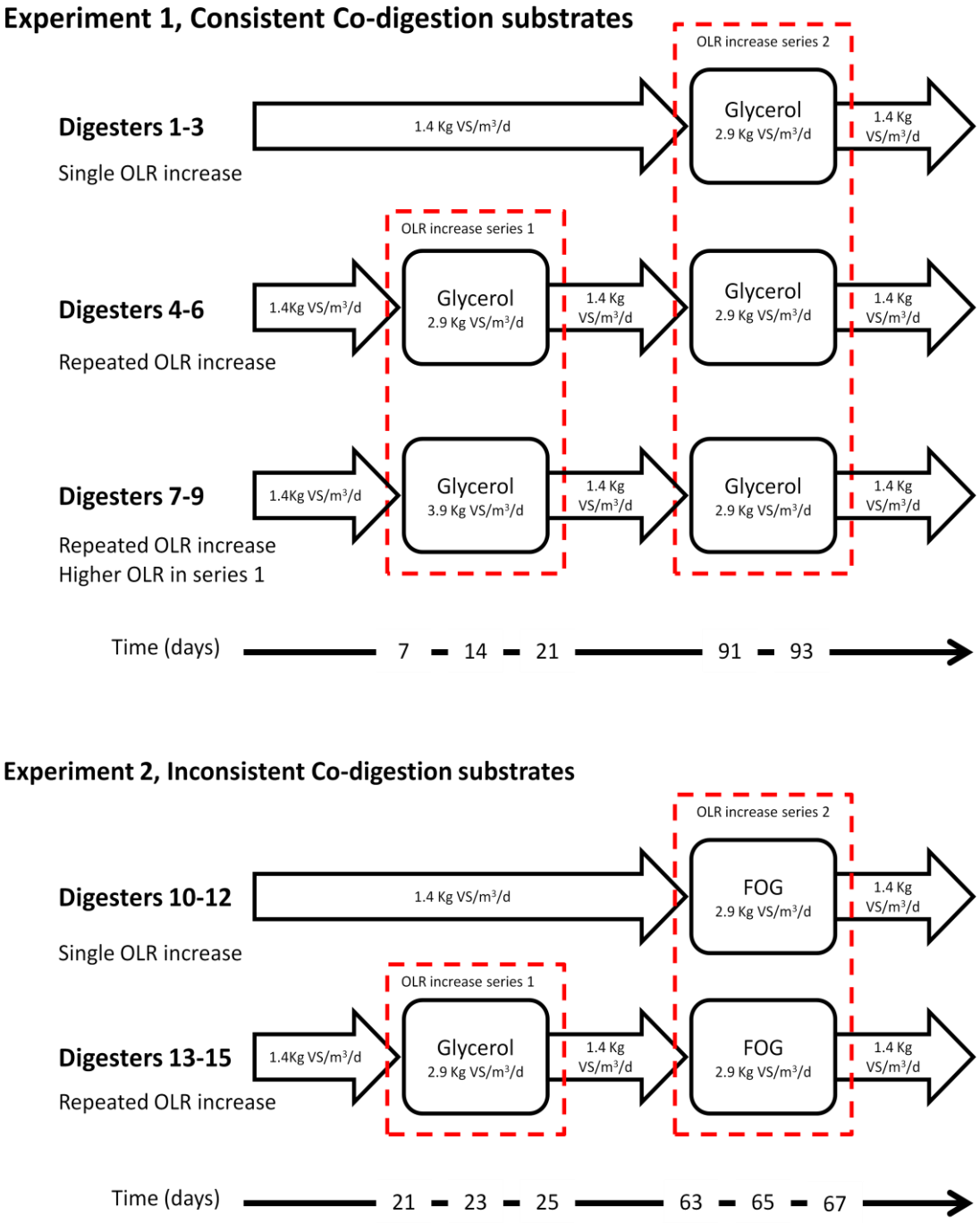


Figure 2.1. Schematic of experimental procedure. All conditions were tested in triplicate.

Table 2.1. Summary of experimental conditions tested

				Start up			OLR increase serries 1				Recovery	OLR increase serries 2				Recovery
Condition	Digesters	Digester working volume (mL)	HRT (days)	Days	OLR	Feedstock	Days	OLR	Feedstock	Additional feedstock concentration (g/L)	Days	Days	OLR	Feedstock	Additional feedstock concentration (g/L)	Days
Experiment 1																
Single OLR increase	1	700	7	0-6	1.4	PS	-	-	-	-	22-90	91, 93	2.9	PS + glycerol	30	94-137
	2	700	7	0-6	1.4	PS	-	-	-	-	22-90	91, 93	2.9	PS + glycerol	30	94-137
	3	700	7	0-6	1.4	PS	-	-	-	-	22-90	91, 93	2.9	PS + glycerol	30	94-137
Repeated OLR increase	4	700	7	0-6	1.4	PS	7, 14, 21	2.9	PS + glycerol	30	22-90	91, 93	2.9	PS + glycerol	30	94-137
	5	700	7	0-6	1.4	PS	7, 14, 21	2.9	PS + glycerol	30	22-90	91, 93	2.9	PS + glycerol	30	94-137
	6	700	7	0-6	1.4	PS	7, 14, 21	2.9	PS + glycerol	30	22-90	91, 93	2.9	PS + glycerol	30	94-137
Repeated OLR increase Higher OLR in series 1	7	700	7	0-6	1.4	PS	7, 14, 21	3.9	PS + glycerol	50	22-90	91, 93	2.9	PS + glycerol	30	94-137
	8	700	7	0-6	1.4	PS	7, 14, 21	3.9	PS + glycerol	50	22-90	91, 93	2.9	PS + glycerol	30	94-137
	9	700	7	0-6	1.4	PS	7, 14, 21	3.9	PS + glycerol	50	22-90	91, 93	2.9	PS + glycerol	30	94-137
Experiment 2																
Single OLR increase	10	4500	7	0-20	1.4	PS	-	-	-	-	26-62	63, 65,67	2.9	PS + FOGs	1.5	68-120
	11	4500	7	0-20	1.4	PS	-	-	-	-	26-62	63, 65,67	2.9	PS + FOGs	1.5	68-120
	12	4500	7	0-20	1.4	PS	-	-	-	-	26-62	63, 65,67	2.9	PS + FOGs	1.5	68-120
Repeated OLR increase	13	4500	7	0-20	1.4	PS	21, 23, 25	2.9	PS + glycerol	30	26-62	63, 65,67	2.9	PS + FOGs	1.5	68-120
	14	4500	7	0-20	1.4	PS	21, 23, 25	2.9	PS + glycerol	30	26-62	63, 65,67	2.9	PS + FOGs	1.5	68-120
	15	4500	7	0-20	1.4	PS	21, 23, 25	2.9	PS + glycerol	30	26-62	63, 65,67	2.9	PS + FOGs	1.5	68-120

2.2.2 Feedstock characterisation

Three substrates were used in the two experiments. Primary sludge collected from a Sewage Treatment Works (Milton Keynes, UK), glycerol waste collected from biodiesel manufacture, and FOGs waste collected from a restaurant grease trap (Cranfield University, UK). Three combinations of substrate were used, primary sludge alone and primary sludge in co-digestion with glycerol waste or FOGs waste. Characterisation of the feedstocks are given in Table 2.2

Table 2.2. Summary of feed and seed stock composition. Triplicate average error bars show standard deviation.

Characteristic	Unit	Seed	Primary sludge	Co-digestion		
Co-digestion substrate				glycerol waste + PS		FOGs waste + PS
Co-digestant concentration	$g\ l^{-1}$			30	50	1.5
OLR	$m^3\ kg^{-1}$ VS day^{-1}		1.4	2.9	3.9	2.9
pH		7.73 ± 0.05	7.09 ± 0.00	7.40 ± 0.04	7.43 ± 0.03	7.53 ± 0.1
TS	%	4.59 ± 0.46	1.46 ± 0.56	2.34 ± 0.43	2.53 ± 1.65	2.33 ± 1.36
VS	% of TS	63.17 ± 0.04	65.93 ± 0.13	88.38 ± 2.31	91.66 ± 3.42	97.82 ± 1.0
sCOD	$g\ l^{-1}$	127 ± 0.65	42.0 ± 1.45	84.46 ± 0.97	115.65 ± 0.62	141.43 ± 3
Alkalinity	$g\ l^{-1}\ CaCO_3$	5.5 ± 0.5	2.5 ± 0.7	2.3 ± 0.1	2.4 ± 0.6	2.1 ± 1.2

2.2.3. Biogas production, methane concentration and physicochemical characterisation

Gas production was measured daily by water displacement in a glass column (150 x 5 cm, green food dye was used to aid measurement). Gas volume was corrected to standard ambient temperature and pressure (SATP) (25 °C and an absolute pressure of 100 kPa). Methane content was measured using a SERVOPRO1400 CH_4 gas analyser (Servomex, UK) according to manufacturer recommendations. pH and alkalinity were measured according to standard APHA methods (APHA 1989). V/A ratio was calculated as the ratio between total VFA (measured by HPLC analysis) and total alkalinity measured according to standard APHA methods. Propionate:Acetate (P:A)

ratio was calculated as the ratio between propionic and acetic acid concentration measured by HPLC analysis, a ratio of greater than 1.4 is considered an indicator of imminent digester failure (Hill *et al.* 1987).

2.2.4. Volatile fatty acids analysis

A 40 ml aliquot of the digestate was centrifuged at 5000 g for 5 min and the supernatant was filtered to < 0.45 μm with a syringe filter. 5 μl of 97 % sulfuric acid was added (to avoid acid degradation when stored) and the sample was stored at – 20 º C until analysis. 100 μl of the sample was injected into a HPLC (535 Kontron, Bio-TEK, UK) equipped with a Bio-Rad fermentation column (Cat 125-0115) 300 x 7.8 mm maintained at 65ºC, and a UV detector at 210 nm. The mobile phase was 0.001 M sulphuric acid in HPLC grade water with a flow rate of 0.8 ml/min. Acetic, propionic, n-butyric, iso-butyric and lactic acids were quantified using an external multilevel calibration ranging from 0.1 g l^{-1} to 5 g l^{-1}. The % error in the repeatability of measurements for each acid was 0.6, 0.77, 0.72, 1.13, and 3.35 % respectively.

2.2.5. Statistical analysis

Statistical analysis was carried out using the R project for statistical computing (http://www.R-project.org/). Analysis of variance (ANOVA) with multiple error terms was used to test for significant differences between means and to account for pseudo replication from repeated measures on the same digester. Analysis of covariance (ANCOVA) was used to test for differences between the correlations of V/A Ratio and biogas methane content when different feedstock. Significance was accepted at $p < 0.05$.

2.3. Results and discussion

2.3.1. Effect of a single OLR increase series on AD performance using glycerol waste or FOGs waste as a co-digestion substrate

Digesters were exposed to single OLR increase series (increase series 2) with glycerol waste (digesters 1-3) or FOGs waste (digesters 10-12) as a co-digestion substrate at an OLR of 2.9 kg VS m^{-3} d^{-1}. OLR increase with glycerol co-digestion resulted in an immediate decrease in biogas production and methane content (to less than 0.01 m^3 kg^{-1} VS day $^{-1}$ and CH_4 < 20 %, Figure 2.2a). In contrast, OLR increase with FOGs initially resulted in an increase in biogas methane content by 7 % to > 80 % and the biogas production by 20 % to 0.1 m^3 kg^{-1} VS day $^{-1}$ (63-69 Figure 2.2b). After day 70, the biogas production decreased by two orders of magnitude and the % of methane decreased to < 30 %. Biogas production recovered after 45 ± 0 and 52 ± 1 days in the glycerol and FOGs waste treatments respectively. After recovery from glycerol waste co-digestion the biogas production was 17 % higher (significant at $p < 0.01$, ANOVA) while methane content was unaffected at 70 ± 4 % (Table 2.3). This indicates that although the total productivity of the microbial community is the same (biogas production) the amount of VS processed into methane has increased, indicating an optimisation of the methane production pathway. In the digesters co-digesting FOGs both biogas production and methane content decreased significantly by 42 and 14 % respectively (Table 2.3). These results show that although FOGs initially increased the methane production the longer term functioning of the digester was affected. In contrast glycerol waste, which immediately inhibited methane production, seemed to improve methane production in the long-term. These results demonstrate that the ongoing function of AD must be considered when optimising AD as the full picture is not

obtained in short studies and biomethane potential tests (BMP) this is addressed in section 2.2.2.

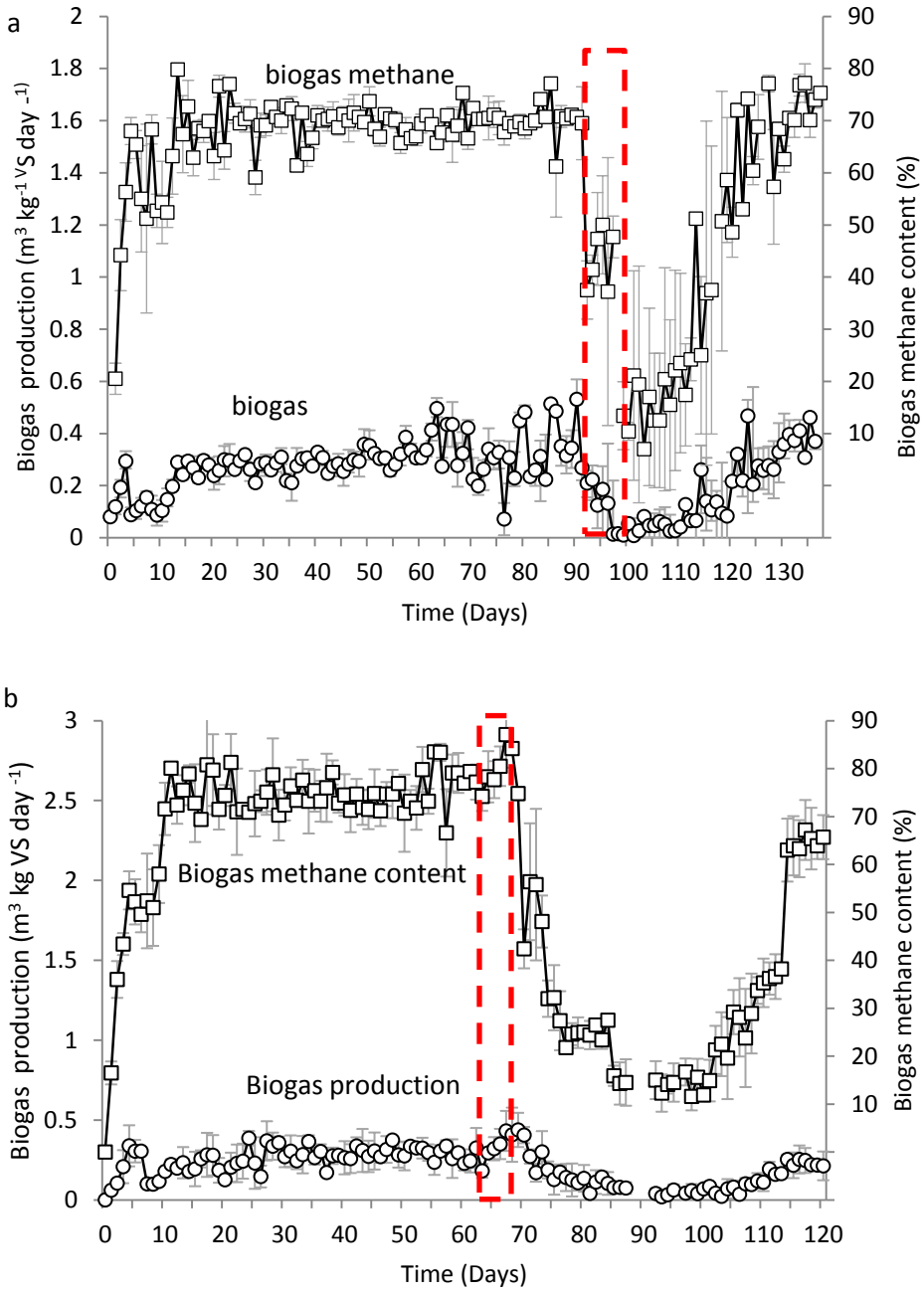


Figure 2.2. Biogas production (circles) and biogas methane content (squares) in (a) digesters 1-3, co-digesting glycerol waste and (b) digesters 10-12, co-digesting FOGs waste. Triplicate digester average, error bars represent standard deviation. Red boxes indicate OLR increase series.

Table 2.3. Average biogas production (m^3 kg^{-1} VS day^{-1}) and methane content (%) before co-digestion and after recovery from co-digestion of primary sludge with glycerol waste or FOGs waste.

	Glycerol waste (digesters 1-3)		FOGs waste (digesters 13-15)	
units	Methane %	Biogas production m^3 kg^{-1} VS day^{-1}	Methane %	Biogas production m^3 kg^{-1} VS day^{-1}
Before co-digestion	69 ± 7[a]	0.29 ± 0.08[a]	74 ± 5.4[a]	0.27 ± 0.09[a]
After recovery	70 ± 4[a]	0.34 ± 0.11[b]	60 ± 12[b]	0.19 ± 0.07[b]

Following the decrease in biogas production and methane content pH decreased from > 7 to < 6.5 and alkalinity values decreased from of 2.8 ± 0.3 g to 1.2 ± 0.1 $CaCO_3$ l^{-1} in digesters co-digesting glycerol waste (digesters 1-3) (Figure 2.3a). This was accompanied by an increase in V/A ratio to > 3 by day 93 rising to 7.7 on day 97 (Figure 2.3, panel a). In the digesters co-digesting FOGs waste (digesters 13-15) the alkalinity and pH was initially unaffected, which is consistent with the increase in biogas production and methane content (Figure 2.3b). However, the V/A ratio increased to 1.6 ± 0.4 indicating accumulation of VFA and demonstrating that this parameter can be used to predict digester upset (Figure 2.3b). On day 74 (as biogas production decreased) there was a decrease in pH to < 6 and alkalinity (from 3.3 ± 0.2 to 1.6 ± 0.8 g $CaCO_3$ l^{-1}), this was parallel to a further increase in the V/A ratio value to > 7. In the glycerol waste co-digestion the pH started to recover immediately after it reached its lowest value on day 97 (the day after the last addition) while in the FOGs co-digestion pH, alkalinity and V/A ratio did not start to return to pre-addition levels until 45 days after the last FOGs addition. This indicates that inhibition from FOGs has a longer-term

effect on AD which is reflected in the lower biogas production and biogas methane content after recovery (Figure 2.2, Table 2.3).

These results show that V/A ratio is a practical method for predicting process failure as when FOGs was used to increase OLR, despite an initial increase in biogas production and stable pH and alkalinity there was an increase in V/A ratio that predicted the process failure which was observed after day 67. Alkalinity and the V/A ratio are very easy parameters to monitor manually, by the operator, or continuously on-line. A cheap and simple titration can be used to estimate alkalinity and VFA concentration which can then be used to calculate the Ripley ratio (which is similar to V/A ratio but based on titration rather than HPLC); they have therefore been suggested as suitable process control parameters for anaerobic digesters (Ripley *et al.* 1986; Feitkenhauer *et al.* 2002; Lahav and Morgan 2004). A Ripley ratio value around 0.25 has been identified as typical of stable AD, however we observed higher values for V/A ratio in stable reactors (Ripley *et al.* 1986). In this study V/A ratio ranged between 0.1 and 1 during stable conditions with an average of 0.34, an increase in the V/A ratio by more than an order of magnitude indicated AD upset. A possible explanation for this discrepancy is the way VFA concentration was measured. The use of HPLC to determine VFA concentration provides a more accurate value than titration. Quantification of VFA by titration only estimates about 65 % of the total VFA with a decrease in accuracy as VFA concentration increases, accounting for this would bring our average value during stable conditions under the threshold of 0.25 (Lahav and Morgan 2004). It is therefore possible that V/A and Ripley ratio will always be correlated with performance when OLR is changed, as shown by (Sanchez *et al.* 2005; Rincón *et al.* 2007) but that the specific reference values may vary for different digester conditions.

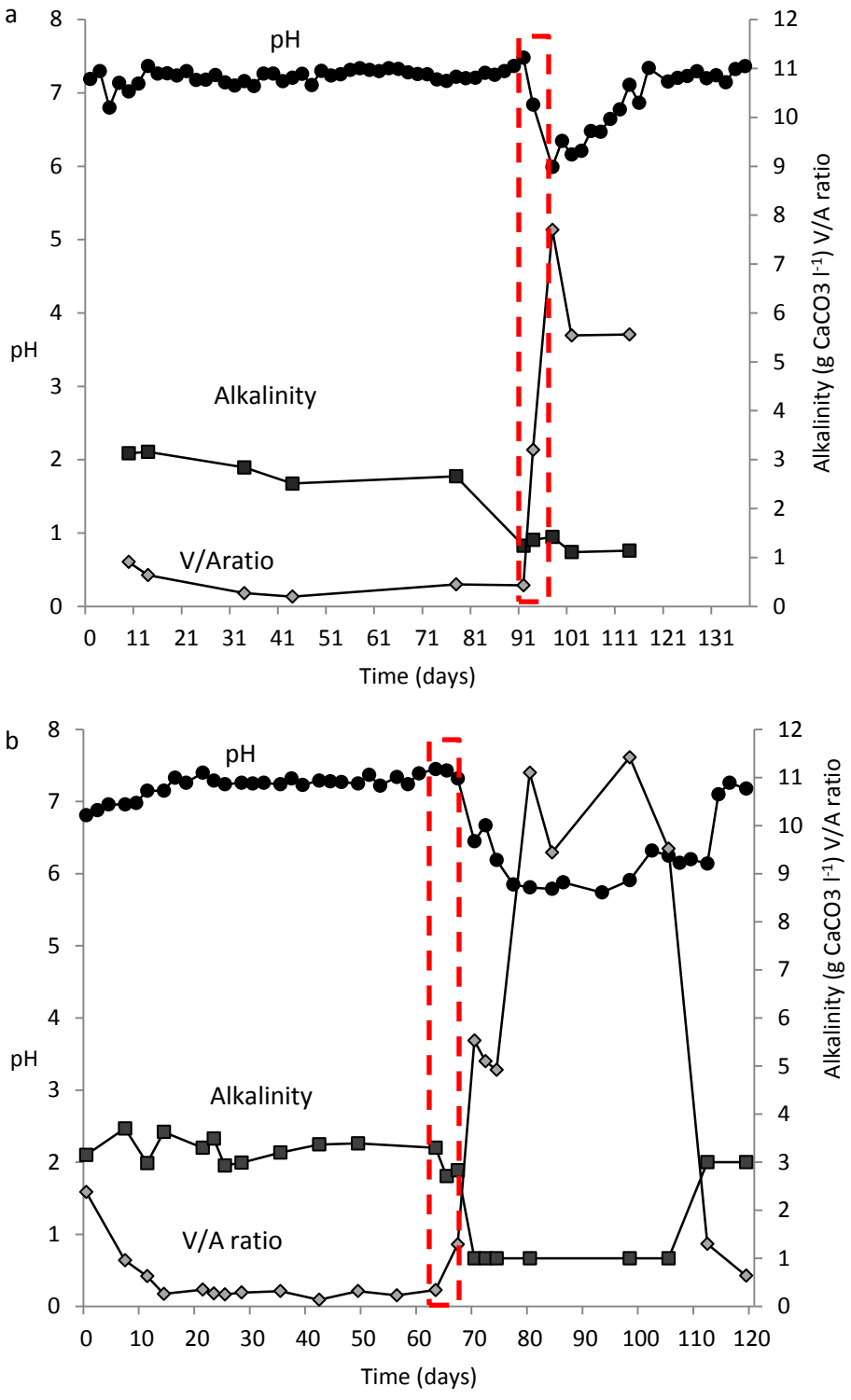


Figure 2.3. pH (black circles) alkalinity (dark grey squares) and V/A ratio (light grey circles) in (a) digesters 1-3, co-digesting glycerol waste and (b) digesters 10-12, co-digesting FOGs waste. Triplicate digester average, % variation for pH = 1.1, alkalinity = 17, and V/A ratio = 23. Red boxes indicate OLR increase series.

The P:A ratio has been previously identified as a parameter that can be used to predict process failure in overloaded anaerobic digesters as it changes in advance of other parameters such as pH and biogas output (Hill *et al.* 1987; Marchaim and Krause 1993). A threshold value of > 1.4 has been reported as indicative of poor digester performance (Hill *et al.* 1987; Marchaim and Krause 1993). In this study OLR change during glycerol waste co-digestion (digesters 1-3) did not result in an increase of P:A ratio to > 1.4 but it did result in a smaller significant increase ($p = 0.02$, ANOVA) from 0.29 to 0.71. Although absolute VFA concentration increased by an order of magnitude, the production of acetic and propionic acids was approximately equal (in parallel) resulting in only a small increase of their ratio (Figure 2.4a). For monitoring purposes this indicates that it may be more suitable to monitor the full picture of the VFA rather than the P:A ratio only. In contrast, P:A ratio was highly dynamic in digesters 13-15 during co-digestion of FOGs waste. P:A ratio initially decreased from 0.7 to 0.2 due to the high concentrations of acetic acid (Figure 2.4b) and then increased sharply to > 1.4 by day 85 when propionic acid became the dominant VFA produced. This increase would predict impending digester failure; however by this point methane content was already low (< 30 %). These results indicate that using P:A ratio for process monitoring cannot provide enough information as the ratio values might not change in advance of changes in methane production. The results also indicate that AD operators will be required to determine threshold values for P:A ratio based on their specific operation. P:A ratio may be related to the specific digestion pathways associated with the different co-digestion substrates, a more detailed analysis of the VFA fingerprints, including other AD fermentation products such as lactic acid and butyric acid is presented in chapter 4 as a fuller picture is required to fully exploit VFA data for AD optimisation.

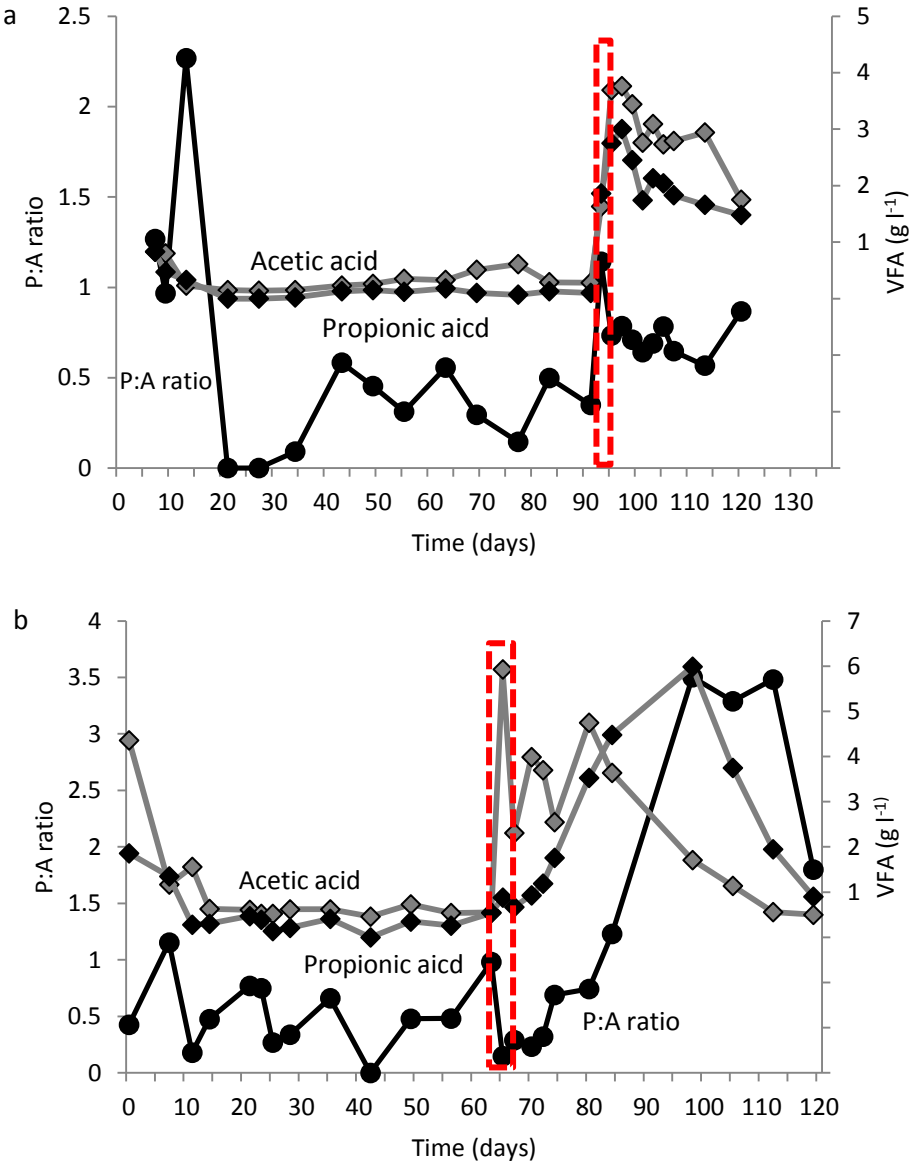


Figure 2.4. P:A ratio (black circles) acetic acid concentration (gray diamonds) and propionic acid concentration (black diamonds) in (a) digesters co-digesting glycerol waste and (b) digesters co-digesting FOGs waste (panel b). Triplicate digester average, % variation for P:A ratio = 43 acetic acid = 26 % and propionic acid = 48 %. Red boxes indicate OLR increase series.

2.3.2. Effect of repeated OLR increase series on AD performance using one or two co-digestion substrates.

Two OLR increase series were carried out using glycerol waste co-digestion for increase series 1 (2.9 kg VS m^{-3} d^{-1},digesters 4-6 and 13-15) followed by glycerol waste co-digestion (digesters 4-6) or FOGs waste co-digestion (digesters 13-15) at 2.9 kg VS m^{-3} d^{-1} during increase series 2. OLR increase series 1 (glycerol waste) caused an immediate decrease in biogas production and methane content to < 0.01 m^3 kg^{-1} VS day^{-1} and CH_4 < 30 %, with a parallel decrease in pH and alkalinity (pH < 6, alkalinity < 1) and an increase in V/A ratio to > 4 (Figure 2.5 and Figure 2.6). For OLR increase series 2 in the digesters exposed to consistent co-digestion substrate (4-6) the biochemical parameters showed the same responses noted for increase series 1, except for the V/A ratio value which reached a higher max value of 9. The digester exposed to a inconsistent co-digestion substrate (digesters 13-15) responded in a different way after increase series 2, showing biochemical patterns more similar to the ones observed in digesters 10-12 (single OLR increase series co-digesting FOGs - section 2.3.1) rather the one recorded after increase series 1. The V/A ratio values were also much higher than the one calculated for the first OLR increase. The average biogas production and methane content were significantly affected by the repeated OLR increases. When consistent co-digestion substrate was used (digesters 4-6) the biogas production doubled and methane content increased by 7 % after the first change. Whereas when inconsistent different substrates where used (digesters 13-15) methane content decreased by 18 %. This has implications for digester operators as it suggests that they should avoid sudden changes in feedstock composition as this may result in reduced performance in terms of biogas production and quality. It also indicates some adaptation in the biomass to

specific co-digestion substrates. Microbial adaptation to specific substrates will be covered in chapter 3, where the microbial communities will be examined in an attempt to understand the links between feedstock changes, microbial adaptation and AD optimisation.

The effect of a higher OLR of 3.9 kg VS m^{-3} d^{-1} (corresponding 50 g l^{-1} glycerol waste) during OLR increase series 1 was also investigated. During increase series 1 performance in terms of biogas production and methane content was not different to that of the lower OLR (corresponding 30 g l^{-1} glycerol waste) Figure 2.5. However the V/A ratio was one third higher reflecting greater accumulation of VFA due to the higher OLR (Figure 2.6). Previous research has shown that higher numbers of bacteria associated with degradation of VFA after OLR increase is responsible for improved resilience to OLR increase (Stroot *et al.* 2001; McMahon *et al.* 2004). Therefore a higher OLR increase in increase series 1 may result in improved/changed performance during subsequent OLR increase series. During increase series 1 performance in terms of biogas production and biogas methane content was not different between the two treatments, however parameters related to VFA production were changed. The pH did not decrease to < 6 during OLR increase 2, the max concentration of acetic acid was doubled to 6 mg l^{-1} and the V/A ratio was approximately 40 % lower (Figure 2.6 and Figure 2.7). Therefore the processing of glycerol waste to acetic acid was more efficient in digesters 7-9, however this was not reflected in improved methane yields or shorter recovery times.

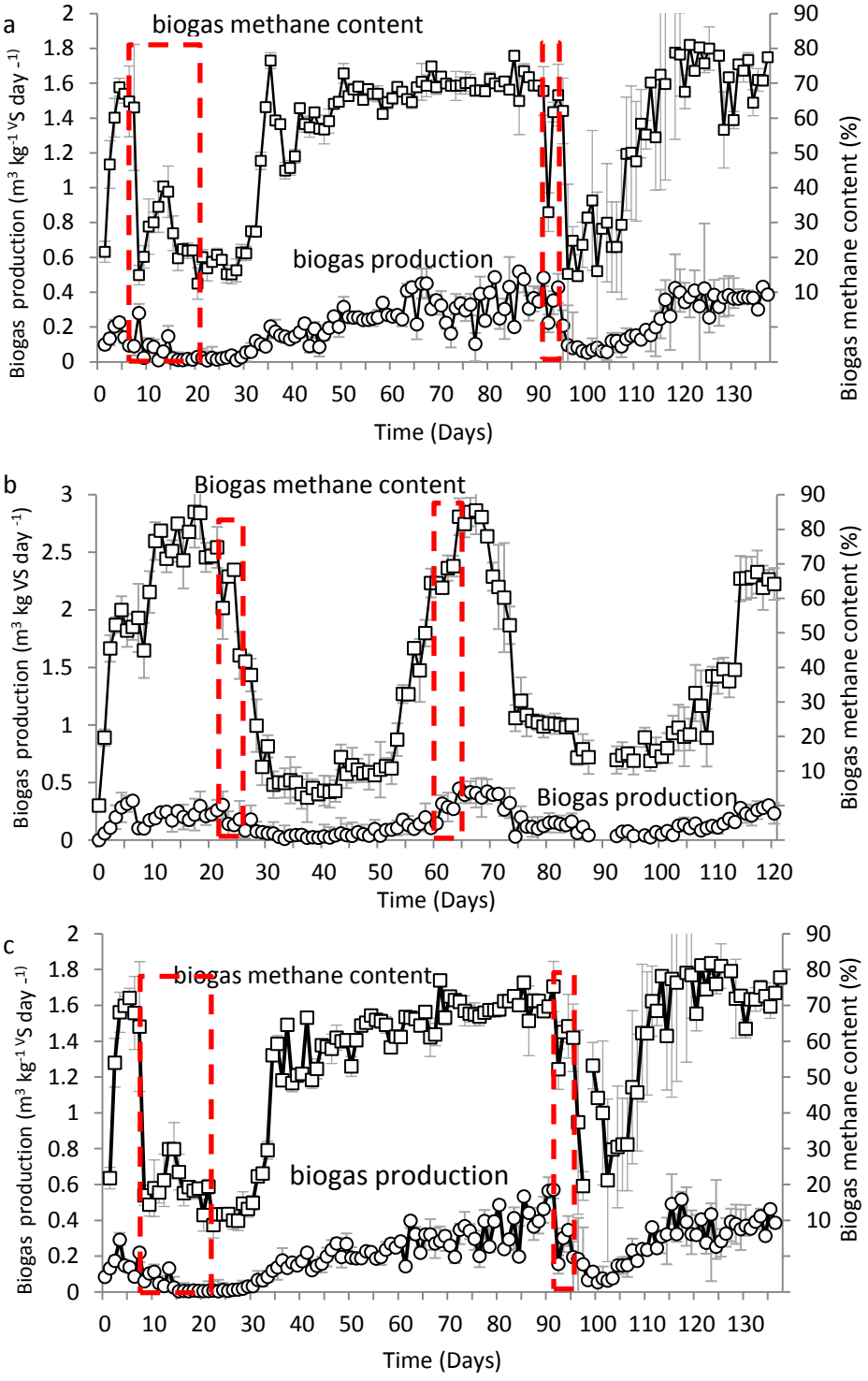


Figure 2.5. Biogas production (circles) and biogas methane content (squares) in digesters exposed to multiple OLR increase series with (a) consistent substrates, (b) inconsistent substrates, and (c) consistent substrates but with different OLRs during the first increase series. Triplicate digester average, error bars represent standard deviation. Red boxes indicate OLR increase series.

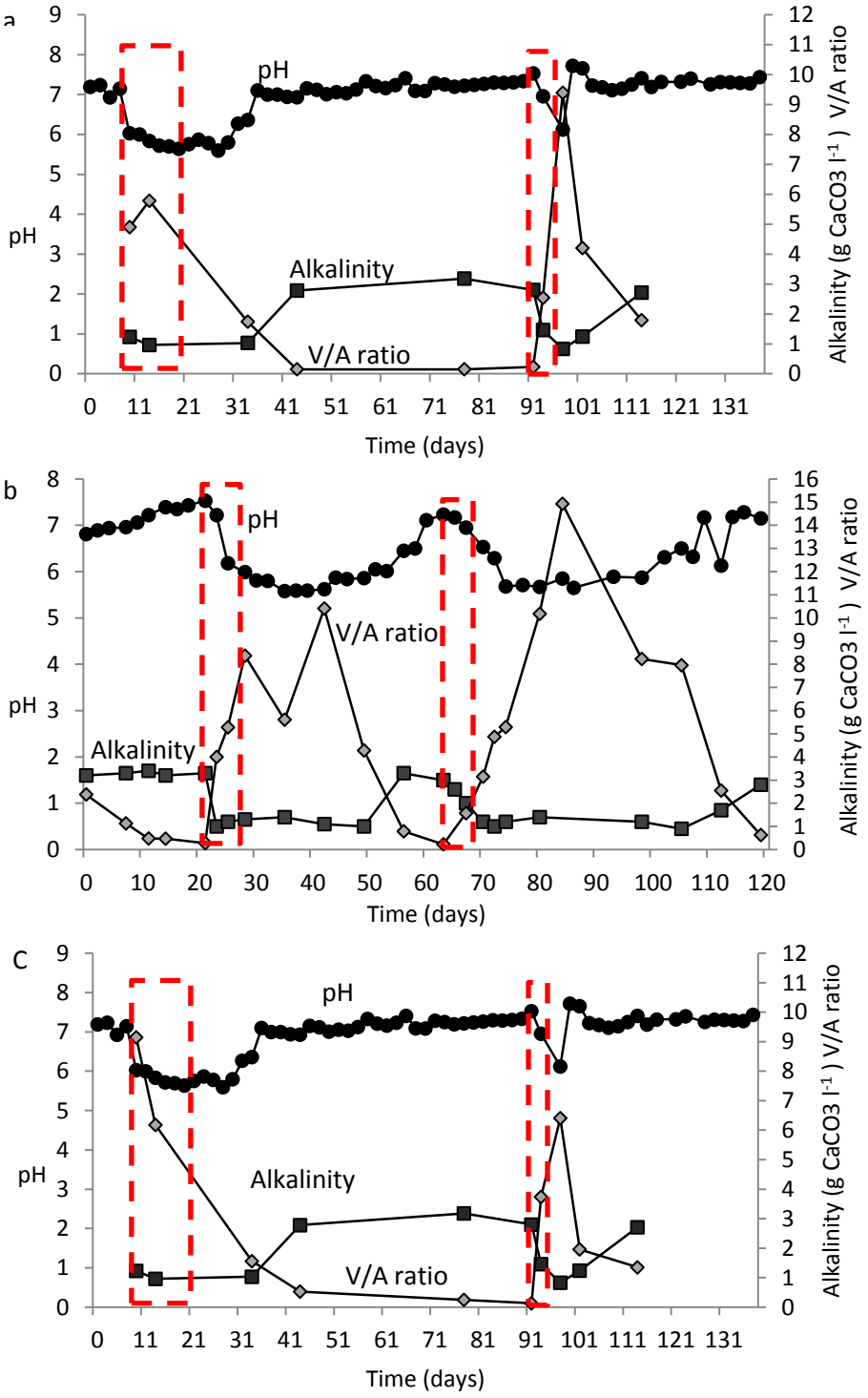


Figure 2.6. pH (black circles) alkalinity (dark grey squares) and V/A ratio (light grey circles) in digesters exposed to multiple OLR increase series with (a) consistent substrates, (b) inconsistent substrates, and (c) consistent substrates but with different OLRs during the first increase series. Triplicate digester average, % variation for pH = 2, alkalinity = 17, and V/A = 25. Red boxes indicate OLR increase series.

Table 2.4. Average biogas production (m^3 kg^{-1} VS day^{-1}) and methane content (%) before the first OLR increase series, after the first OLR increase series and after the second OLR increase series. In digesters exposed to multiple OLR increase series with consistent or inconsistent feedstock. Different superscript letters in same column notes significant differences.

Feedstock	Consistent		Inconsistent	
Digesters	4-6		13-15	
Parameter	Methane	Biogas production	Methane	Biogas production
Units	%	m^3 kg^{-1} VS day^{-1}	%	m^3 kg^{-1} VS day^{-1}
Before 1st increase series	66 ± 5^a	0.17 ± 0.08^a	76 ± 7^a	0.21 ± 0.09^a
After 1st increase series	67 ± 5^b	0.32 ± 0.1^b	73 ± 15^a	0.18 ± 0.12^a
After 2nd increase series	73 ± 8^b	0.36 ± 0.05^b	57 ± 13^b	0.20 ± 0.08^a

Digester recovery from OLR series 2. The digesters that were exposed to both OLR increase series (digesters 4-9) recovered to initial methane content and biogas production levels significantly faster than the digesters that were only exposed to increase series 2 (digesters 1-3) (Table 2.5). For biogas methane content the recovery was 8-11 days (approximately 1.5 HRT) faster and for biogas production 15-18 days (2-2.5 HRT) faster than digesters 1-3. There was however no significant difference in recovery times between digesters 4-6 and 7-9 which were exposed to different OLR during increase series 1. These results demonstrate that tolerance of OLR increase was improved in digesters after exposure to past OLR increase series. This is in agreement

with McMahon *et al.* (2004) but the results reported here provide a much more robust analysis due to the use of triplicate replication.

Table 2.5. Times recorded for biogas production (m^3 kg^{-1} VS day^{-1}) and methane content (%) to revert to initial values after a single or a multiple OLR increase. Different superscript letters in same column notes significant differences.

Digesters	methane	Biogas production
Units	%	m^3 kg^{-1} VS day^{-1}
1-3 – after one OLR increase	31 ± 1^a	45 ± 0^a
4-6 – after two OLR increase	23 ± 3^b	30 ± 6^b
7-9 – after two OLR increase	20 ± 2^b	27 ± 1^b

Analysis of the VFA profiles shows that this may be related to changes in the way the feedstock were processed and the resultant production of VFA. In digesters 4-6, the production of VFA during increase series 2 was different than for increase series 1, with acids produced in parallel for the first increase series and in sequence for the second (propionic and then acetic acid) (Figure 2.7, panel a). This resulted in a far more dynamic P:A ratio during OLR increase series 2, specifically an initial increase to > 1 followed by a decrease to < 0.4 and finally an increase to > 1.5 as the dominance of the two VFA changed. This indicates that the processing of VFA changed over the long-term operation of the digesters over multiple OLR increase series resulting in more efficient recovery from inhibition of methanogenesis. Previous studies have shown that changes in the way VFA are processed, either a greater diversity of VFA processing pathways can result in improved performance (Hashsham *et al.* 2000; McMahon *et al.* 2004). In both cases this was linked to differences in the microbial community structure (Fernández *et al.* 2000; Stroot *et al.* 2001; McMahon *et al.* 2004). Therefore the changes

in VFA profile reported in this study may be linked to adaptation or shifts in the biomass which will be investigated in Chapters 3 and 4.

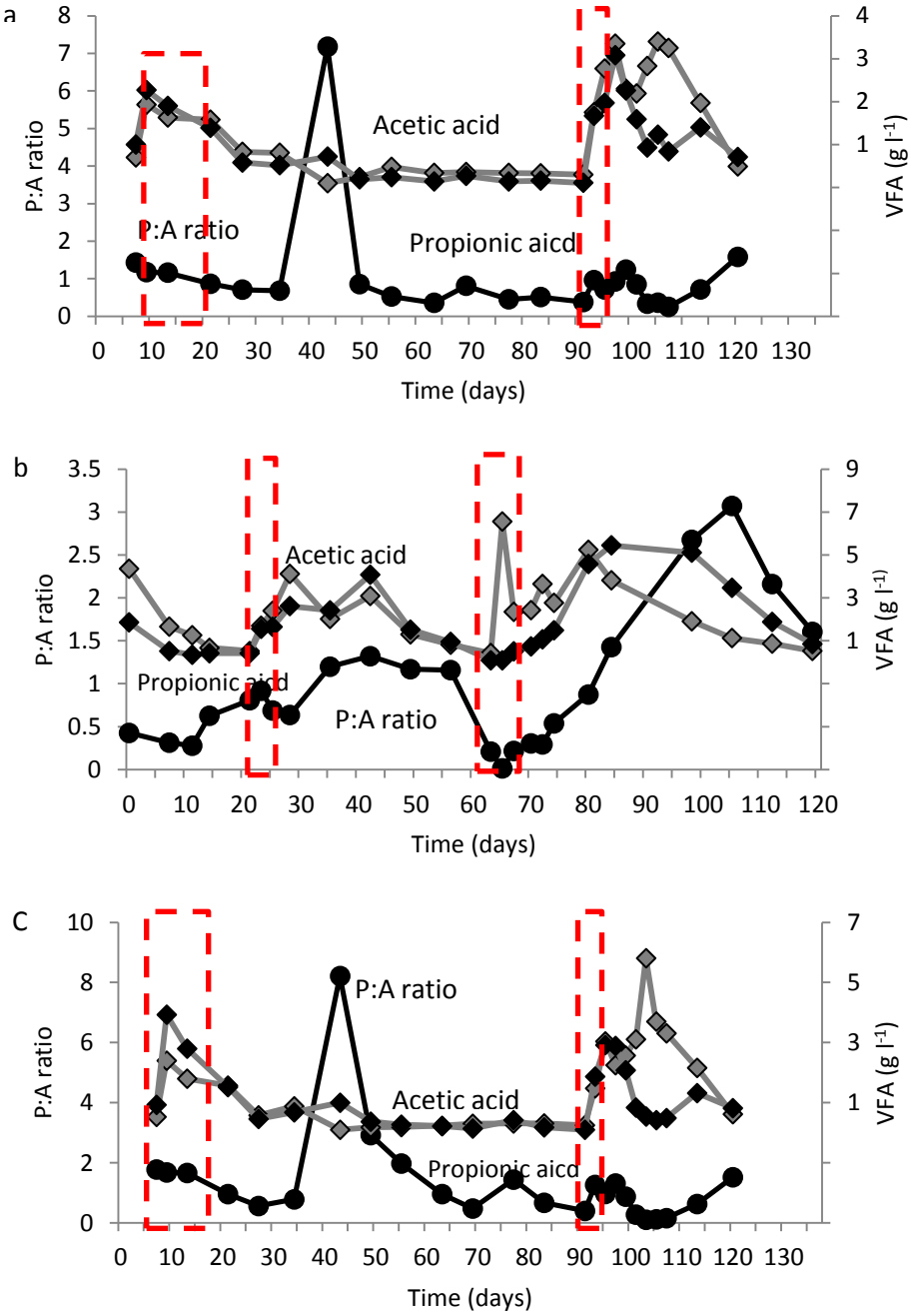


Figure 2.7. P:A ratio (black circles) acetic acid concentration (grey diamonds) and propionic acid concentration (black diamonds) in digesters exposed to multiple OLR increase series with (a) consistent substrates, (b) inconsistent substrates, and (c) consistent substrates but with different OLRs during the first increase series. Triplicate digester average, % variation for P:A ratio = 43 %, acetic acid = 25 % and propionic acid = 32 %. Red Boxes indicate OLR increase series.

2.3.3. Implications for operational monitoring and optimisation of AD

Multiple changes in OLR and feedstock composition: The results reported in this chapter demonstrate that digesters pre-exposed to OLR increase series recover to initial values of biogas quantity and quality faster (Table 2.5) when exposed to additional OLR increase series. They also show that multiple changes in OLR using the same feedstock can produce and increase in AD performance in terms of biogas quantity and quality (Table 2.4). This is in agreement with results presented by McMahon and Zheng *et al.* (2004) Chen *et al.* (2012) and Palatsi and Laureni *et al.* (2009) who showed that digesters pre-exposed to increases in OLR exhibited improved performance. Palatsi and Laureni *et al.* (2009) showed that digesters exposed to repeated LCFA pulses had faster recovery times when exposed to LCFA again, and that this was linked to adaptation rather than changes in the microbial groups present in the reactor (Palatsi *et al.* 2010). McMahon and Zheng *et al.* (2004) showed that digesters exposed to high OLR during start up developed distinct microbial communities with increased numbers of syntrophic groups and showed improved performance during a much higher organic overload increase series than the one use in this study (18.8 kg VS m^3 day^{-1}). The links between reactors performance improvement and changes in bacterial community structure and microbial metabolic pathways will be covered in the following chapters (chapter 3 and 4 respectively).

The analysis of the biochemical parameters has also shown that inconsistent feedstock composition caused a decrease in AD performance. This clearly has implications for AD operators and suggests that it is important to maintain stable feedstock composition to maximise long-term methane yields which may be hard as feedstock availability and competition can vary throughout the year (Akunna *et al.* 2007).

Monitoring of AD: The results reported in the section 2.3.3 clearly demonstrate that the V/A ratio value is a better indicator of AD performance than the P:A values. In all the conditions tested the V/A ratio values varied much more readily than the P:A values showing an increase well before recordable changes in biogas quantity and quality were measured. This is also demonstrated by linear regression of the parameters against biogas methane content, for P:A ratio the R^2 is < 0.01 indicating hardly any variation in biogas methane content can be predicted from P:A ratio. In contrast regression with V/A ratio gives and R^2 of 0.6 and $p < 0.001$, demonstrating that V/A ratio is a good predictor of biogas methane content. Feedstock composition seems to have an influence on V/A ratio values, as they were approximately double during inhibition caused by FOGs co-digestion than for glycerol waste after the second OLR change. ANCOVA did not detect any significant change in the slope ($p = 0.6$) of the regression of biogas methane content and V/A ratio for the two feedstocks but it did indicate a lower intercept ($p = 0.04$, Figure 2.8). This shows that reference values for V/A ratio are affected by feedstock but that the nature of the correlation is constant. As discussed in section 2.3.1 Ripley ratio is similar to V/A ratio as both are a measure of buffering capacity of the system and Ripley ratio may be the more suitable than V/A ratio for AD operators due the ease of calculation. Values for Ripley ratio may be different to the V/A values presented in this study and are higher than those reported in the literature (excluding values of 17 reported by McMahon *et al.* (2004) for the reasons outlined in section 2.3.1. The Ripley ratio was first developed using AD of poultry manure as reference; in this system the critical parameter is ammonia concentration rather than VFA. Thus care must be taken when using feedstocks/co-digestion substrates that lead to high VFA concentration as this may result in poor estimation of Ripley ratio (Ripley

et al. 1986; Pechan *et al.* 1987; Bujoczek *et al.* 2000). It is therefore reasonable to assume that as AD opens to the treatment of new feedstocks the operator's reference values for Ripley ratio will need to be re-assessed and established for each plant and for each operation.

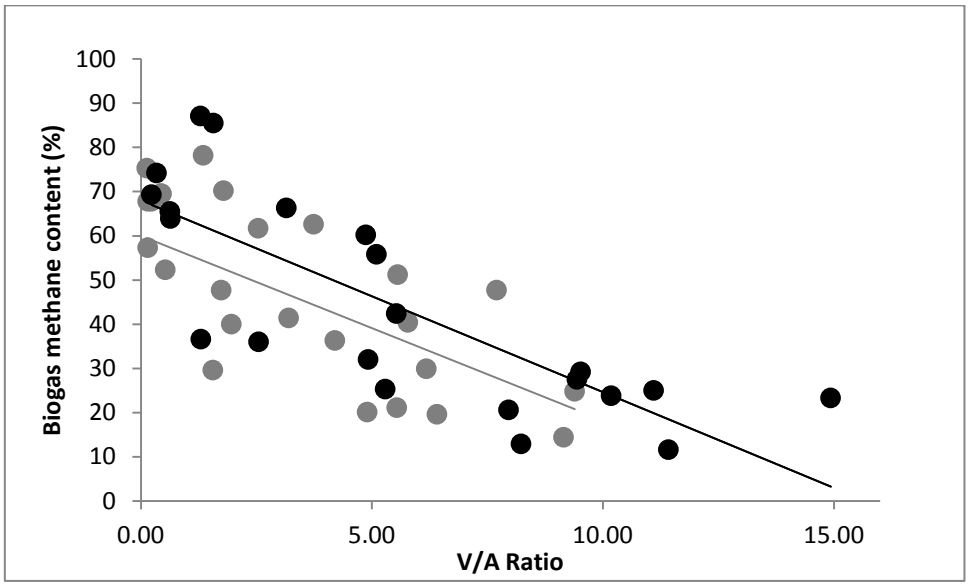


Figure 2.8. Scatter plot of Biogas methane content and V/A Ratio. Grey dots represent glycerol waste co-digestion and black FOGs waste. Lines represent linier regression, for glycerol waste slope = -5.1, intercept = 59, R^2 = 0.57 and for FOGs slope = -4.3, intercept = 67, and R^2 =63. ANCOVA showed no significant difference between slope of regression (p = 0.6) but intercepts were significantly different (p = 0.04).

The results of the chapter related to the P:A ratio have also shown that this monitoring parameter is an inconsistent and unreliable indicator for AD performance. The values did not show any changes during glycerol waste co-digestion whereas during FOGs waste co-digestion the ratio changed some time after the variation in AD performance. This can be easily explained by the fact that it is the decrease in pH and not the VFA content and composition that directly inhibit digester performance (Gourdon and Vermande 1987) and that the pH in AD is controlled by both alkalinity and VFA. Despite this, knowledge of the P:A value can provide information on the changes in the

way the different VFA are processed in digesters during OLR increase. The P:A values could be used to identify specific digestion pathways associated with the different feedstocks/co-digestion substrates to provide indication on recovery times. To further understand the implications of this ratio, a more detailed analysis of the VFA fingerprints is presented in chapter 4 where changes in acids are associated to changes in operational parameters and bacterial groups.

3.4. Conclusions.

The results in this chapter demonstrate that short-term trials with single changes in conditions and/or BMP tests do not provide a full picture of how a feedstock will perform in AD in realistic operational conditions. Multiple OLR increase series using the same co-digestion substrate resulted in significant improvements in performance (biogas production doubled and methane content increased by 7 %) and recovery times (1.5 HRT faster after the second OLR increase series). Further work (Chapter 3) will investigate if this is due to changes in the microbial community structure, and or metabolic function. In contrast multiple OLR increase series with inconsistent feedstocks resulted in a decrease in performance and no change in recovery times.

V/A ratio was shown to be the most consistent indicator of performance in comparison to P:A ratio, pH, alkalinity and biogas production. However values for both parameters varied depending on feedstock composition indicating that values for these ratios will depend on the specific operational conditions of individual digesters.

Despite the inconstancy in P:A ratio changes in the way VFA were processed was linked to the faster recovery observed in digesters exposed to multiple OLR increase series with glycerol waste, therefore, a fuller picture of how VFA are processed in AD is required to understand how to optimise the technology (Chapter 4). A diagram

summarising critical parameters in terms of performance (biogas methane content) based on our results is presented below (Figure 2.9). In Chapter 5, a detailed microbial analysis investigates to provide information on the shifts in the microbial community that can be added to this schematic and used for AD monitoring.

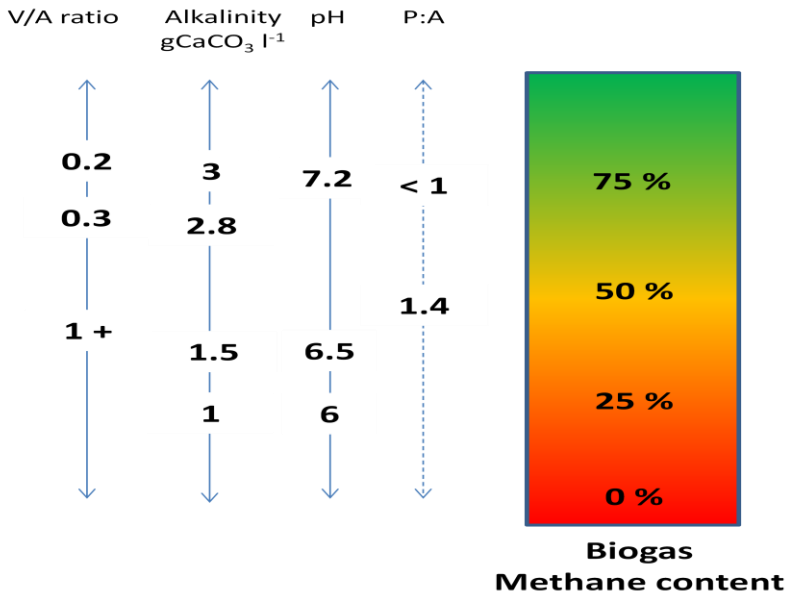


Figure 2.9. Summary of critical parameters in terms of performance (biogas methane content) based on our results, A:P ratio is given in dashed line as it is inconsistent.

Chapter 3: The Effect of feedstock and OLR changes on microbial community during co-digestion

Abstract: The effect of two different co-digestion substrates, glycerol waste and FOGs waste in co-digestion with primary sludge, on microbial community was evaluated during single and multiple increase series of organic loading rate (OLR). Both co-digestion substrates caused a 5 and 15-fold decrease in bacterial and archaeal biomass respectively accompanied by a decrease in biogas methane content to < 30 %. During the single OLR increase series there was a high proportion of candidate phylum OP10 (26 %) in digesters co-digesting FOGs waste, in contrast the digesters co-digesting glycerol waste had higher proportions of *Clostridia Incertae Sedis* XV (38 %) and *Ruminococcaceae* (17 %). Further to this, *Firmicutes* members doubled in both treatments as biogas methane content decreased. Increase in *Firmicutes* and decrease in archaeal biomass was also seen when digesters were exposed to multiple increase series in OLR with glycerol waste and FOGs waste confirming that these parameters could form the basis of microbial monitoring of AD. In chapter 2 it was demonstrated that anaerobic digesters pre-exposed to a OLR increase series can have shorter recovery times when exposed again to OLR increase. In this chapter this was linked to changes in the microbial community response at family level, specifically a higher proportion *Clostridia Incertae Sedis* XV in the pre-exposed digesters. It is speculated that higher the numbers of *Clostridia Incertae Sedis XV* (related to *Synergistaceae Cloacibacillus*) in the pre-exposed digesters decreased digester recovery times as it resulted in syntrophic interactions with archaea. Therefore this group should be considered for bioaugmentation in unstable AD.

3.1. Introduction

In Chapter 2 it was shown that digesters pre-exposed to OLR increase series were more resilient to further increases in OLR with the same co-digestion substrate. In this chapter the aim is to understand if this can be linked to the microbial response, and evaluate if this could be applied to AD optimisation.

Co-digestion can be used to optimise the digestion of many organic wastes, including the organic fraction of municipal solid wastes (OFMSW), long chain fatty acids (LCFA), sewage sludge, algae, cattle manure, waste milk, corn stalk and vermicompost (Callaghan *et al.* 1999; Sosnowski *et al.* 2003; Fernández *et al.* 2005; Chen *et al.* 2010a; Chen *et al.* 2010b). Wastewater sludge solids and high strength organic wastes have traditionally been used for feedstocks in AD but there is increasing interest in processing new feedstocks. It is therefore important to fully understand the effects of feedstocks composition on the process as co-digestion can have both synergistic and antagonistic effects on AD (Misi and Forster 2001). Although a great deal of research has been carried out to develop tools for co-digestion optimisation based mainly on physical parameters (Derbal *et al.* 2009; Ãlvarez *et al.* 2010) it remains important to understand how the complex consortium of bacteria and archaea in the digesters can be affected by changes in feedstock composition and/or OLR change, as both are well known causes of digester instability.

A number of studies have confirmed that factors such as feedstock composition and OLR can affect the structure of both bacterial and archaeal communities (Dearman *et al.* 2006; Wang *et al.* 2009a; Supaphol *et al.* 2011; Xia *et al.* 2011). However, results between studies are not consistent with some studies showing the structure of the bacterial population as the most affected and relevant in the digestion process and others

showing the opposite (Dearman *et al.* 2006; Zhang *et al.* 2010; Xia *et al.* 2011). Meanwhile researchers have also reported a large number of unidentified bacterial strains and *Crenarchaeota* in AD, the latter in particular, are non-methanogenic archaea with an unclear function in the AD process (Wang *et al.* 2009a; Zhang *et al.* 2010). This highlights how little we still currently understand about the structure and function of microbial communities in AD. The structure and function of microbial communities in optimisation process is a subject that cannot be ignored as several studies have shown a relationship between microbial community structure and AD performance. For example, Zhang *et al* (2010) showed that robust stable archaeal populations were correlated to stable performance whereas Dearman *et al* (2006) established a relationship between methane production rate and bacterial community structure. In addition adaptation of bacterial communities to changes in OLR during co-digestion have been shown to improve recovery times after periods of instability, both by changes in community structure (higher numbers of syntrophic propionate-oxidizing and fatty acid-beta-oxidizing bacteria) and changes in physiology (McMahon *et al.* 2004; Palatsi *et al.* 2010). These studies demonstrate that it is not sufficient to investigate the effect of single changes in conditions on AD performance and that it is important to understand the long-term performance of AD.

The objective of this chapter is to; understand how two different co-digestion substrates a sugar-like feedstock (glycerol waste) and/or a lipid feedstock (fat, oil and greases waste, FOGs) influence the microbial community in when used to increase OLR as co-digestion substrates, to investigate the effect of multiple OLR increase series on the microbial community.

A combination of lipid analysis and 454-pyrosequencing was used to resolve the microbial community of the digesters. The two techniques have different purposes as lipid analysis can detect changes in the physiology of bacteria and Archaea while 454-pyrosequensing provides information on the phylogenetic fingerprint of the community (Frostegård *et al.* 2011).

3.2. Methods

3.2.1. Operational parameters

Operational parameters are given in section 2.2.1

3.2.2. Biogas production, methane concentration

Gas production was measured daily by water displacement in a glass column (150 x 5 cm, green food dye was used to aid measurement). Methane content was measured using a SERVOPRO1400 CH_4 gas analyser (Servomex, UK) according to manufacturer recommendations.

3.2.3. Phospholipids and ether-linked isoprenoids analysis

Total lipids were extracted from 40 g aliquot of freeze-dried digestate using a modified version of the Bligh-Dyer technique as described by Frostegård, *et al.* (2011). The dried fatty acid methyl esters (FAMEs) were resuspended in 0.2 ml of hexane and analysed by gas chromatography equipped with flame ionisation detector (GC-FID Agilent Technologies 6890N) as described by Pankhurst *et al.* (2012). FAMEs were identified by comparison of retention times with the 26 bacterial acid methyl ester (BAME) mix standard (SUPELCO, Sigma, UK). Nonadecanoic acid methyl ester (Sigma, UK) was added (24.44 $\mu g\ ml^{-1}$) as an internal standard to each sample after solid phase extraction (SPE).

Another aliquot of the phospholipids fraction, equivalent to 40 g of the digestate was used for PLEL analysis according to the method described by Gattinger, *et al.* (2003). The dried ether-linked isoprenoids were reconstituted in 0.2 ml of hexane and analysed by gas chromatography coupled to mass spectrometry (GCMS Agilent Technologies 6890N) according to the operating conditions described by Gattinger, *et al.* (2003). Nonadecanoic acid methyl ester (Sigma, UK) was added as an internal standard to each sample after SPE.

3.2.4. Taxonomic affiliation of PLFA and PLEL

The taxonomic affiliations are summarised in Table 3.1. Gram-positive bacteria were represented by the series of iso and anteiso branched saturated PLFA. Gram-negative bacteria were represented by cyclopropane, hydroxyl and monounsaturated PLFA. The 16:0 straight chain PLFA has been previously demonstrated as an ubiquitous bacterial marker (Piotrowska-Seget and Mrozik 2003). The PLFA 18:2w9cis and 18:1w7trans used as markers for clostridia. The PLEL i20:0 was used as a marker for the E*uryarchaeota*, i20:1 as a marker of the aceticlastic methanogens belonging to *Methanosarcina* and i40:0 as a marker for hydrogenotrophic methanogens belonging to *Methanobacterium, Methanococcus, Methanopyrus, and Methanothermus* (Gattinger *et al.* 2002; Radl *et al.* 2007). Bacterial biomass was converted into number of cell equivalents using a conversion factor of 5.9×10^{10} cells per µmol of PLFA (Kieft *et al.*, (1994) and archaeal biomass was estimated using a conversation factor of 5.9×10^{13} cells per 2.5 µmol PLEL (Bai *et al.*, 2000).

Table 3.1. Summary of PLFA and PLEL taxonomic affiliation (adapted from Gattinger *et al.* 2002; Gattinger *et al.* 2003; Londry *et al.* 2004; Oravecz *et al.* 2004; Radl *et al.* 2007).

		Taxonomic affiliation
Straight saturated		
11:0		
12:0		
		δ-Proteobacteria
13:0		
14:0	ubiquitous	*Actinobacteria/ δ-Proteobacteria*/ low GC G+
		Actinobacteria
15:0		
16:0		*Actinobacteria*/ low GC G+/CFB/ *δ-Proteobacteria*
17:0		δ-*Proteobacteria*
18:0		low GC G+ *Bacillus/ Clostridium*
20:0		
iso and anteiso branched		
iso-15:0		low GC G+/*Bacillus/ Actinobacteria*
anteiso-15:0	G+	*Actinobacteria*/ *Bacillus*/ low GC G+/CFB[a]/ *Proteobacteria*
iso-16:0		low GC G+/ *Bacillus/ Actinobacteria*
iso-17:1		CFB (*Cytophaga*)/SRB[b]
cyclopropane		
cyc 17:0	G-	Anaerobes/ *Bacillus/ Clostridium* / δ-P*roteobacteria*
cyc-19:0		Anaerobes/ *Bacillus/ Clostridium* / δ-*Proteobacteria*
mono-unsaturated		
16:1 w7cis		*Bacillus/ Clostridium*
18:2 w6cis	G-	*Actinobacteria* / *Bacillus/ Clostridium*
18:1 w9cis		Anaerobes/ *Bacillus/ Clostridium*
18:1w9trans		Anaerobes/ *Bacillus/ Clostridium*
poly-unsaturated		
18:2w6,9		Fungi
hydroxy		
2OH-10:0		*δ-Proteobacteria*
		δ-Proteobacteria
2OH-12:0		
	G-	*δ-Proteobacteria*
3OH-12:0		
3OH-14:0		*δ-Proteobacteria*
2-OH 16:0		*δ-Proteobacteria*/CFB
isoprenoids		
i20:0	Methanogens	*Euryarchaeota*
i20:1		*Methanosarcina*
i40:0		Hydrogenotrophic methanogens

[a] CFB: *Cytophaga-Flavobacteria-Bacteroides* [b]SRB: sulphate-reducing bacteria

3.2.5. 454-pyrosequencing analysis

A 40 g aliquot of the digestate was collected using aseptic techniques and stored at -80ºC until analysis. 200 mg of the sample was used for DNA extraction using a MoBio Power Soil kit (MO BIO Laboratories, Inc, UK) and the quality of DNA was checked on 0.8% agarose gels. For the amplification of different 16S rRNA gene fragments a high fidelity polymerase (Phusion, Biolabs, New England, UK) was used according to the manufacturer instructions. For amplification of the bacterial 16S rRNA gene fragments PCR primers were adapted for 454 amplicon sequencing by attaching the M13 adapter (highlighted yellow) to the target forward primer, namely for bacteria M13-16S-IA-FL (5'-CACGACGTTGTAAAACGACCATGCTGCCTCCCGTAGGAGT-3'), whereas the 25-mer Lib-L specific sequence adapter B (highlighted red) was followed by the reverse template specific primer sequence 16S-IA-RL (5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGAGAGTTTGATCCTGGCTCAG -3'). To aid multiplexing different samples, different barcodes were included in the M13 adapter (highlighted yellow) using the 454 sequence adapter A (highlighted blue) and a 454 amplicon sequencing specific 4-mer amplification key (highlighted turquoise) followed by a 10-mer barcode sequence (NNNN) (5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNCACGACGTTGTAAAA CGAC-3').

The amplification of the archaeal 16S rRNA gene fragment was carried out using primers ARC-344F (5'-CACGACGTTGTAAAACGAACGGGGYGCAGCAGGCGCGA) and ARC-915R (5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGGTGCTCCCCCGCCAATTCCT-

3') which were adapted for multiplexing and 454 sequencing as described above. Each 20 µL PCR mixture contained primers at 10 µM, 10 mM deoxynucleoside triphosphates and 0.2 µL of High fidelity polymerase (Phusion, Biolabs, New England, UK), 4 µL Phusion 5x buffer (Phusion, Biolabs, New England, UK) and 1.4 µL $MgCL_2$. Amplifications were performed using a Biorad C1000 Thermal cycler (BioRad) as follows: 95 ° C for 5 min, followed by 35 cycles of denaturation at 98 °C for 20 sec, annealing at 57°C for bacterial and 55°C for archaeal amplicons for 20 sec and elongation at 72 °C for 30 s. Cycling was completed by a final elongation at 72 ° C for 10 min. Next generation sequencing (NGS) of all amplicons was completed using the GS FLX System (Roche). Emulsion PCR was carried out according to the manufacturer's instructions (Roche). Samples were multiplexed on a 1/8 section of the pyrosequencing plate. Sequencing resulted in a total of 68,434 bacterial sequences and 4,733 archaeal sequences and an average of 726 and 36 sequences per sample, respectively. The obtained sequence data were processed using the Galaxy platform (http://galaxyproject.org/). Sequences analysed were a minimum of 500 bp (mean length average 560 bp). Splitting of sequences into respective samples was carried out using respective barcodes.

3.2.6. Bioinformatics

The obtained sequence data were then processed using the CloVR-16S 1.0 pipeline (http://clovr.org/) according to White *et al.* (White *et al.* 2011) Briefly, poor quality sequences were removed using the Qiime script "plit_libraries.py" (http://qiime.org) using the following parameters (minimum sequence length 100 bp, maximum sequence length 2000 bp, maximum homopolymer length 8, minimum quality score 25, and maximum ambiguous bases 0). The Mothur script "unique.seqs" was then used to

cluster unique sequences and a set of representative sequences was determined. The representative sequences were then searched against the "16S rRNA gold database" to identify putative chimeras using the default parameters,. The chimeric sequences were then excluded from further analysis. Sequences were then clustered, aligned and classified using the Qiime workflow "pick_otus_throigh_otu_table.py". Sequences were clustered into operational taxonomic units (OTUs) with a 97 % nucleotide sequence identity threshold for all reads within a OTU using the Qiime script "pick_otus.py". Representative sequences for each cluster were then selected with "pick_otus.py". Representative sequences were then classified using the Ribosomal database project (RDP) Bayesian classifier (http://rdp.cme.msu.edu/) at Phylum, Class, Order, and Family with a confidence threshold of 0.5, with the script "assign_taxonomy.py". Results presented are the number of sequences assigned to OTUs identified at the respective taxonomic levels.

3.2.7. Statistical analysis

The proportion of sequences assigned to respective microbial Orders and the abundance of the individual lipid markers were used to calculate Bray Curtis Similarity indices (after log transformation of the data). The similarity indices were then used for non metric multi-dimensional scaling (nMDS) ordination and cluster analysis using the Primer software beta version 6 (PRIMER-E Ltd, UK). Linear regression bacterial/archaeal biomass and methane biogas content was then carried out using the R project for statistical computing (http://www.R-project.org/). Biomass was converted into log cells ml^{-1} kg^{-1} VS added as described in 3.2.4 and biogas methane content was arc sine transformed to approximate normal distribution, significance was accepted at $p < 0.05$.

3.3. Results and discussion

3.3.1. Effect of a single OLR increase series on microbial community using glycerol waste or FOGs waste as a co-digestion substrate.

Changes in biomass: Addition of 30 g l^{-1} glycerol waste to feed in the digesters 1 to 3 during OLR increase series 2 immediately induced a 5 and 15 fold decrease of the bacterial and archaeal biomass respectively, accompanied with a decrease in biogas quantity and quality in terms of methane content (Figure 3.1). The archaeal and bacterial biomasses recovered on day 113 which was 10 and 25 days faster than recovery of biogas methane content and biogas production respectively (as shown in Chapter 2). This shows that microbial biomass can recover in advance of noticeable improvements in biogas production and quality (in terms of methane production) and therefore could be used as an early indicator of digester recovery. In contrast to glycerol waste, OLR change using FOGs waste resulted in an initial increase in biogas methane content by 10 % (Chapter 2) and induced only a decrease in the archaeal and bacterial biomasses as biogas quality decreased on days 80 and 98 respectively (Figure 3.1). These results demonstrate that biomass decreased with biogas methane content for both of the co-digestion substrates.

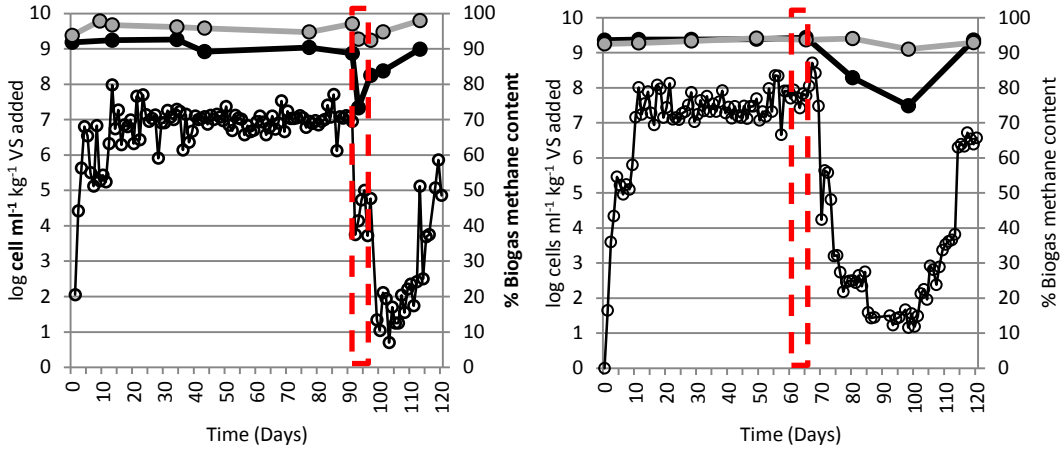


Figure 3.1. Percentage methane (white circles) total bacterial biomass (grey circles) and total archaeal biomass (black circles) in digesters co-digesting glycerol waste digesters 1-3 (left panel) and FOGs digesters 10-12 (right panel) (n = 3, average standard deviation for % methane = 5, bacterial biomass = 0.1, and archaeal biomass = 0.1). Red boxes indicate OLR increase series.

Lipid marker analysis: The dominant PLFAs included 16:0 and 18:0, iso-15:0, anteiso-15:0 (*Actinobacteria*/ low GC G+/CFB/ δ-*Proteobacteria*) 18:1w9trans, 18:1w9cis (Anaerobes and BC group) and iso-17:1 (SRB) (Table 3.1) The PLEL detected were the isoprenoids i20:0 (generic *Euryarchaeota* marker), i20:1 (*Methanosarcina*) and i40:0 (hydrogenotrophic methanogens) (Table 3.1). The only difference in the dominant PLFAs between co-digestion of glycerol waste and FOGs waste was the iso/anteiso-15:0 PLFA, which were lower by 5 % in the FOGs treatment. Although the lipid fingerprint profiles of the all the digesters showed that the microbial community was relatively stable across all conditions tested, there were some noticeable changes occurring immediately after the addition of the co-digestion feedstocks. Specifically there was an increase of the SRB marker iso-17:1 by 10 % and a decrease by 10 % in the PLFA 18:1w9trans day 93 (digesters 1-3) during the glycerol waste co-digestion (Table 3.2). The PLFA iso-17:1 is an indicator of sulphate reduction, which is

known to be an alternative pathway to methanogenesis when syntrophic interactions between bacteria and Archaea are inhibited (Table 3.2); (Van Den Berg *et al.* 1980). In contrast, for the co-digestion of FOGs there were no changes in the proportions of iso-17:1 and 18:1w9trans PLFA (Table 3.2). However the concentration of the isoprenoid i20:0 PLEL (mole %) increased from 45 to 71 % three days after FOGs addition (day 80, during the period when biogas methane content was increased) and then decreased to < 40 % as biogas methane content decreased.

Table 3.2. Mol % of dominant (> 5 %) PLFA and PLEL in digesters co-digesting glycerol waste (digesters 1-3) or FOGs waste (digesters 10-12). Triplicate digester average, Average % error = 6 %. (Only days after OLR increase series are shown). Red borders indicate OLR increase series.

	Glycerol waste					FOGs			
Time (days)	91	93	97	101	113	65	80	98	119
PLFA									
iso-15:0	11	10	11	12	9	5	3	4	2
anteiso-15:0	12	11	11	12	10	4	4	4	4
16:1 w7cis	16	17	17	14	18	10	11	12	10
16:0	15	8	17	14	19	16	15	19	15
iso-17:1	1	12	1	1	1				
18:1 w9cis	12	14	13	13	11	14	14	11	16
18:1w9trans	11	1	6	10	10	16	16	16	20
18:0	9	10	10	10	8	9	13	12	13
PLEL									
i20:1	20	40	4	7	18	8	55	38	55
i20:0	60	60	78	43	16	71	39	21	22
i40:0	20	0	18	50	66	21	6	42	23

454-Pyrosequencing OTUs analysis: Over 75 % of the archaeal diversity identified was related to the genus *Methanosarcina*. Methanogens belonging to the genus *Methanosarcina* are the most stress resistant methanogens and are therefore able to dominate digesters with a wide range of physicochemical conditions (Calli *et al.* 2005a; Calli *et al.* 2005b). Thus it is plausible that *Methanosarcina* did dominate in all conditions tested and that changes in the archaea were only related to biomass and metabolic function.

There were significant differences in the bacterial families present during co-digestion of glycerol waste or FOGs waste during OLR increase series. During the co-digestion of glycerol waste the dominant Clostridia families were *Clostridia Incertae Sedis* XV (38 %) and *Ruminococcaceae* (17 %). The dominant *Bacteroidetes* families were *Porphyromonadaceae* (44 %) and *Rikenellaceae* (22 %) (Figure 3.2). In contrast, during FOGs waste co-digestion 26% of sequences was related to OP10/*Armatimonadetes* and could not be identified to lower than phylum level (Figure 3.3). The closest match to the OP10 OTU was also isolated from an anaerobic digester (JQ114107, 87 %, unpublished). There were also differences in the Clostridia, with *Clostridia Incertae Sedis* XI and *Peptostreptoccaceae* making up 23 and 13%, respectively. These noticeable differences during the early stages of co-digestion for the two substrates show that, despite glycerol waste being an initial product of FOGs oxidation, both feedstocks can be processed through different pathways and require different pool of bacterial groups for AD. This is further investigated in Chapter 4.

Figure 3.2. Taxonomic affiliations of dominant OTUs (> 5%) during co-digestion of glycerol waste in digesters 1-3, day 93 increase series 2 (3 days after start of OLR increase series). Rings show Phylum, Class, Order, and Family moving from inner to outer ring.

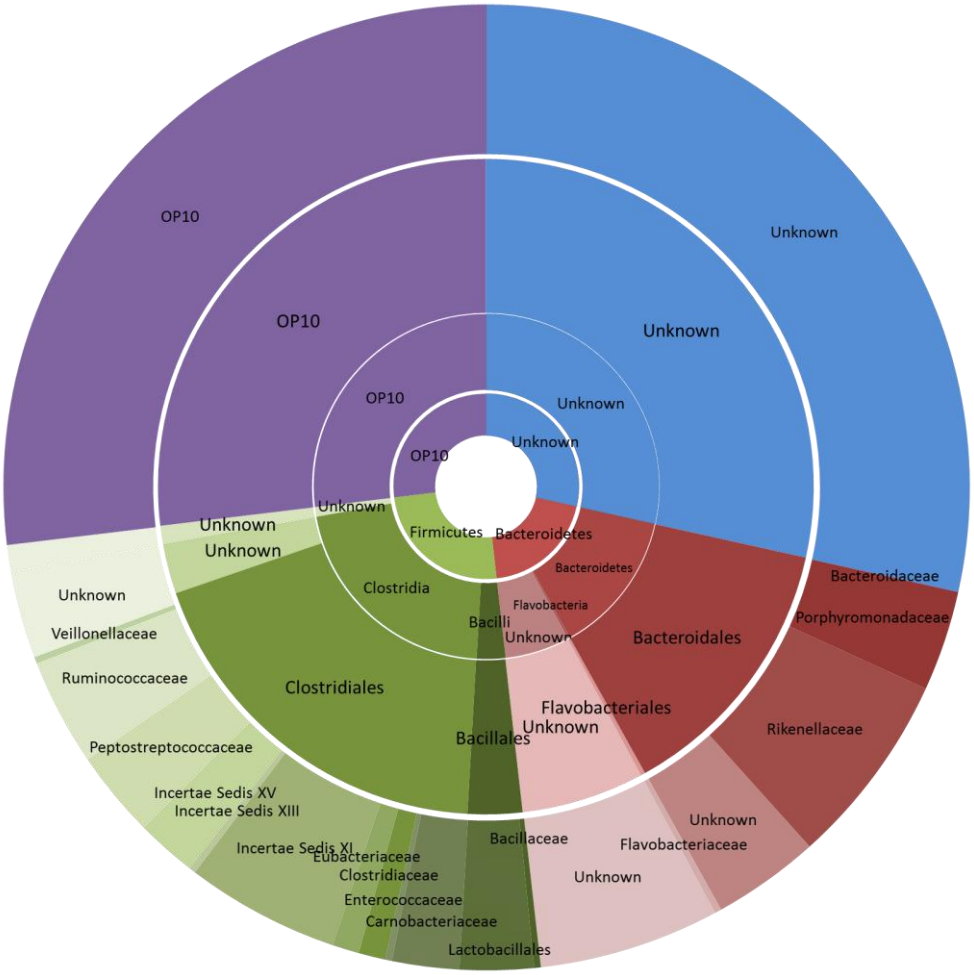
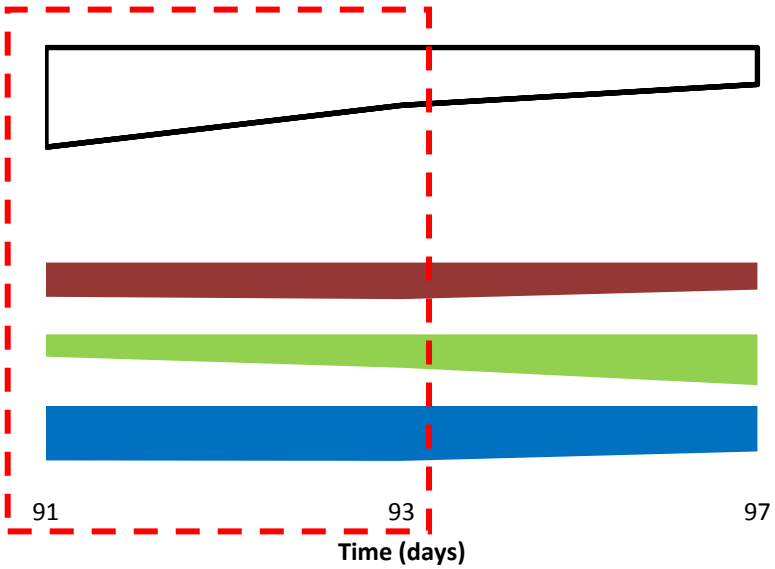


Figure 3.3. Taxonomic affiliations of dominant OTUs (> 5%) during co-digestion of FOGs waste day 65, digesters 10-12 increase series 2 (3 days after start of OLR increase series). Rings show Phylum, Class, Order, and Family moving from inner to outer ring.

Although the initial response of the bacterial community to co-digestion was distinct for the two co-digestion substrates there were similarities in the response of the bacterial community when biogas methane content decreased. 6 days after glycerol waste addition (digesters 1-3, increase series 2) biogas methane content decreased, *Firmicutes* tripled and unknown bacterial OTUs decreased by 20 % (Figure 3.4). For FOGs waste co-digestion biogas methane content did not initially decrease (it increased as discussed chapter 2) but when methane content did decrease there was also a decrease in unknown bacteria and 2-fold increase of *Firmicutes* (day 98). Therefore it can be concluded that the decrease in unknown OTUs and increase in *Firmicutes* OTUs were related to poor AD performance. This is in contradiction with results reported by Rincon *et al.* (2008) where *Firmicutes* were dominant at lower OLR. A probable explanation for this discrepancy is the use of DGGE by Rincon *et al.* (2008) which resolves only few dominant bacterial OTUs. In contrast, in our study the results indicate that there are similarities in the microbial community response to low methane production, irrespective of feedstock/co-digestion. Changes in bacterial community structure and the previously discussed changes in biomass which happen in advance of changes of AD performance, in terms of biogas production and quality could form the basis for monitoring of AD using the technologies discussed in Chapter 1. These results however need to be treated with caution as they are only considering a single OLR increase series and in reality multiple changes in OLR and feedstock composition may occur. In the following section the effect of multiple changes in OLR on the microbial community are investigated.



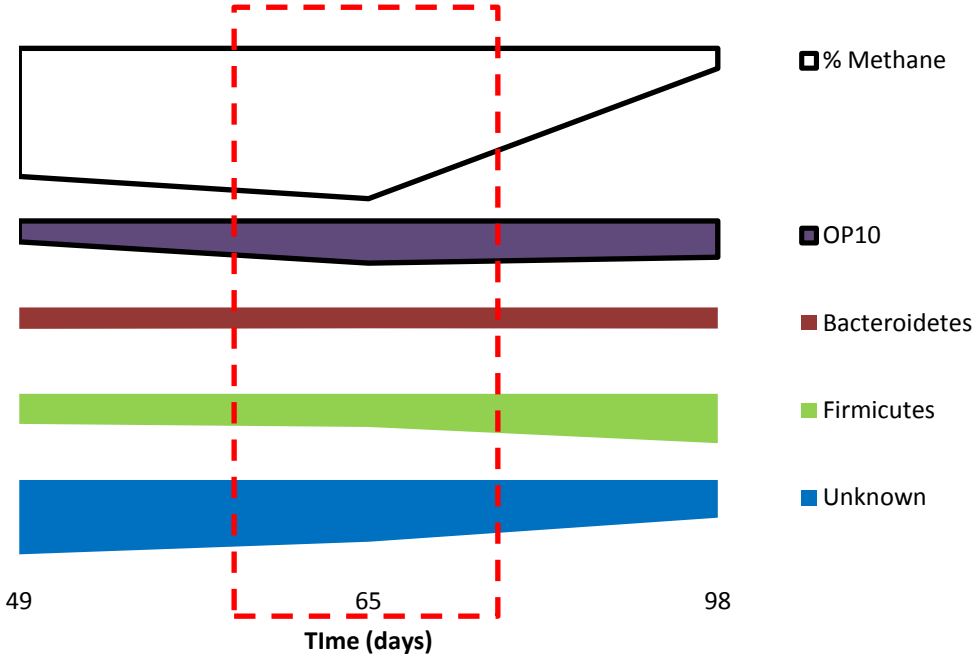


Figure 3.4. Relative changes in bacterial phyla and biogas methane content during glycerol waste co-digestion digesters 1-3 (top panel) and FOGs waste co-digestion digesters 10-12 (bottom panel) based on % of sequences assigned at phyla level. Red box indicates OLR increase series. x axis is not to scale and only shows days when samples were taken.

3.3.2. Effect of repeated OLR increase series on microbial community using one or two co-digestion substrates

In Chapter 2, analysis of the performance parameters of AD showed that digesters pre-exposed to glycerol waste recovered 1.5 HRT faster than the control when exposed to glycerol waste again. Previously Palatsi *et al.* (2010) and McMahon *et al.* (2004) and Chen *et al.* (2012) have shown that increased numbers of key syntrophic groups, adaptation, and increased population redundancy in *Methanosaeta* following substrate overloading can result in optimization of AD to further perturbations such as organic overload. Therefore it is important to understand if this is linked to structural/metabolic changes in the microbial community.

The effects of repeated OLR increase series using glycerol waste co-digestion (increase series 1 and 2, digesters 4-6) on archaeal and bacterial biomass were similar as those observed with a single OLR increase series, a 5 and 15-fold decrease of the bacterial and archaeal biomass respectively (Figure 3.5). The only difference between the two increase series was that during the second OLR increase series the decrease in the archaeal biomass was only noticed 6 days after glycerol waste addition (increase series 2, digesters 4 to 6) which is twice as long as with the single OLR increase series (increase series 2, digesters 1-3). This finding suggests that the methanogen community has acquired resilience to OLR increase with glycerol waste co-digestion which could be linked to shorter recovery observed in Chapter 2. When the co-digestion substrate was changed for the second OLR increase series (switch from glycerol waste to FOGs waste, digesters 13 to 15; increase series 2) the only difference was that bacterial biomass was unaffected, which is in contrast to all other conditions tested (Figure 3.5).

archaeal and bacterial biomasses were able to recover to pre-addition concentrations after both the first and second OLR increase series (Figure 3.5). This demonstrates that multiple changes in OLR can be managed during AD without permanent damage to the microbial community.

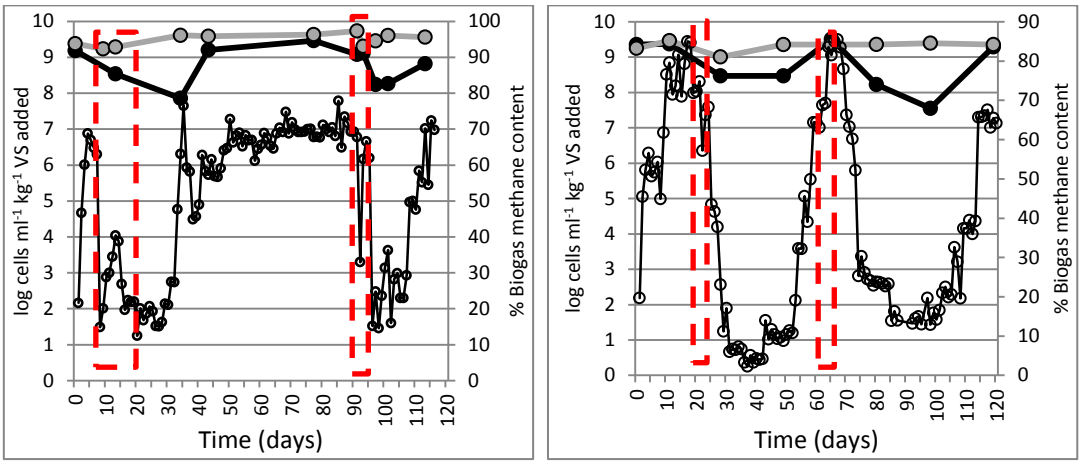


Figure 3.5. Repeated OLR increase series using glycerol waste digesters 4-6 (left panel) or glycerol waste then FOGs waste as co-digestion substrate digesters 13-15 (right panel). Percentage methane (white circles) total bacterial biomass (grey circles) and total archaeal biomass (black circles). (n = 3, average standard deviation for % methane = 5, bacterial biomass = 0.1, and archaea biomass = 0.1). Red boxes indicate OLR increase series.

Lipid marker analysis: The dominant lipid markers were the same as described in section 3.3.1 during the single OLR increase series (digesters 1-3 and 10-12, increase series 2). The main difference between the first and second OLR increase series with glycerol waste was that the PLFA 18:1w9trans was not present on day 9 (digesters 4-6, increase series 1) during the first OLR increase series, but was unaffected in digesters on second (Table 3.3). This is consistent with single OLR increase with glycerol waste (digesters 1-3, increase series 2) where the PLFA 18:1w9trans decreased 10-fold (Table 3.3). When glycerol waste and FOGs waste were used as a co-digestion substrate (digesters 13 to 15, increase series 2) the PLFA 18:1w9cis increased 2-fold after FOGs

co-digestion, with a corresponding decrease of the 18:1w9trans PLFA (Table 3.4). These changes in the Mol % of the 18:1w9cis/trans PLFA (which is related to *Bacillus* or *Clostridia*) indicate that metabolic or structural shifts are taking place within these bacterial groups during OLR increase with both co-digestion substrates, which may be linked to the faster recovery of methane production observed in Chapter 2.

Table 3.3. Mol % of dominant (> 5 %) PLFA and PLEL in digesters co-digesting glycerol waste with repeated OLR increase series (digesters 4-6). Red lines represent OLR increase series.

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	Day										
	0	9	13	34	43	77	91	93	97	101	113
PLFA											
iso-15:0	12	14	11	9	10	10	11	11	8	9	9
anteiso-15:0	10	10	11	10	10	11	10	11	8	10	10
16:1 w7cis	14	16	16	16	16	16	15	15	16	15	15
16:0	19	19	19	19	19	16	14	18	16	17	13
18:1 w9cis	13	13	11	10	12	11	14	11	13	13	14
18:1w9trans	14	0	8	10	11	12	11	10	12	11	13
18:0	5	5	9	9	10	9	8	10	9	9	10
PLEL											
i20:1	33		26	43	23	26	25	16	12	24	11
i20:0	38		64	0	54	59	53	75	67	30	13
i40:0	29		11	57	23	15	22	9	21	46	75

Triplicate digester average, average % error = 5.8 %

Table 3.4. Mol % of dominant (> 5 %) PLFA and PLEL in digesters co-digesting glycerol waste followed by FOGs waste (digesters 13-15). Red lines represent OLR increase series.

	Day							
	0	11	28	49	65	74	98	119
PLFA								
iso-15:0	4	2	2	3	3	4	4	5
anteiso-15:0	4	4	2	3	3	4	5	4
16:1 w7cis	12	10	11	8	10	9	7	7
16:0	16	16	17	19	17	15	10	14
18:1 w9cis	13	16	14	15	15	24	34	13
18:1w9trans	15	19	17	18	16	16	8	18
18:0	8	13	10	11	12	8	9	15
PLEL								
i20:1	40	44	5	44	13	31	25	42
i20:0	51	51	91	10	77	62	40	34
i40:0	9	5	5	46	10	7	34	25

Triplicate digester average, average % error = 6 %.

Comparison of the lipid fingerprint profiles in digesters 1-9 increase series 2 highlighted two distinct groups at 80 % similarity (Figure 3.6 panel a). Group I contains digesters with stable performance while group II contains digesters after the OLR was changed (days 93-97). The only exception to this was the non pre-exposed digesters at day 93 (digesters 1-3) which show a distinct lipid fingerprint structure. This highlights that the bacterial lipid response to glycerol waste OLR increase is different in digesters which have been pre-exposed to an OLR increase series with glycerol waste co-digestion before, further demonstrating that faster recovery (as reported in Chapter 2) is related to adaptation in the bacterial community. Comparison of the bacterial lipid fingerprints in digesters exposed to FOGs waste OLR increase during increase series 2 (digesters 10-15) showed that the bacterial community remain at > 90 % similarity on all days excluding day 98 (Figure 3.6, panel b). Thus demonstrating that the similarity in performance between these two groups observed in Chapter 2 can be related to similarities in the lipid fingerprints in the digesters.

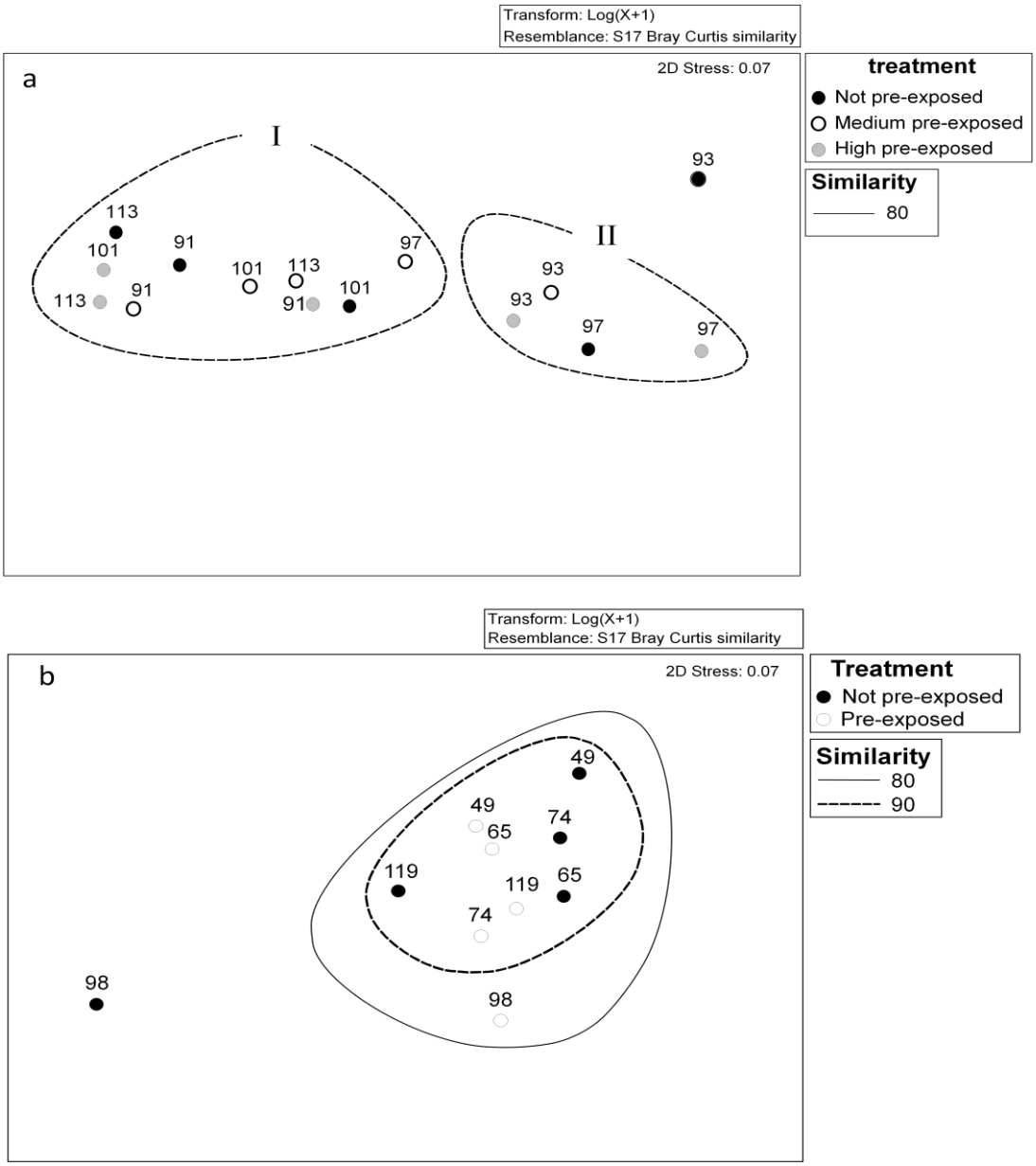

Figure 3.6. Bacterial lipid structure during OLR increase series 2 in digesters exposed to consistent feedstock 1-9 (a) and inconsistent feedstock 10-15 (b). Profile similarities are based on UPGMA cluster analysis using log transformation data and the Bray Curtis similarity indices. Numbers show days as indicated in Figure 2.1. Black circles show digesters only exposed to OLR increase series 2 and white and grey circles show digesters exposed to OLR increase 1. Triplicate digester averages were used for the cluster analysis.

454-Pyrosequencing OTUs analysis: As with the digesters exposed to OLR increase series 2 only (digesters 1-3, 10-12) there was a doubling in *Firmicutes* as biogas methane content decreased in all conditions and after both OLR increase series (Figure 3.7). For repeated addition of glycerol waste OLR increase series, *Bacteroidetes* initially tripled during low biogas methane content (day 9) and then decreased. In contrast for glycerol waste followed by FOGs addition increase series there was a doubling in *Bacteroidetes* at day 65 as biogas methane content increased which was then reversed as biogas methane content decreased (Figure 3.7). These results clearly illustrate the relationship between the *Firmicutes* and the biogas methane content, as observed with single increase series of OLR increase is repeated over multiple OLR increase series. This finding suggests that monitoring for increase *Firmicutes* OTUs during AD could be used to predict poor performance in terms of biogas methane content.

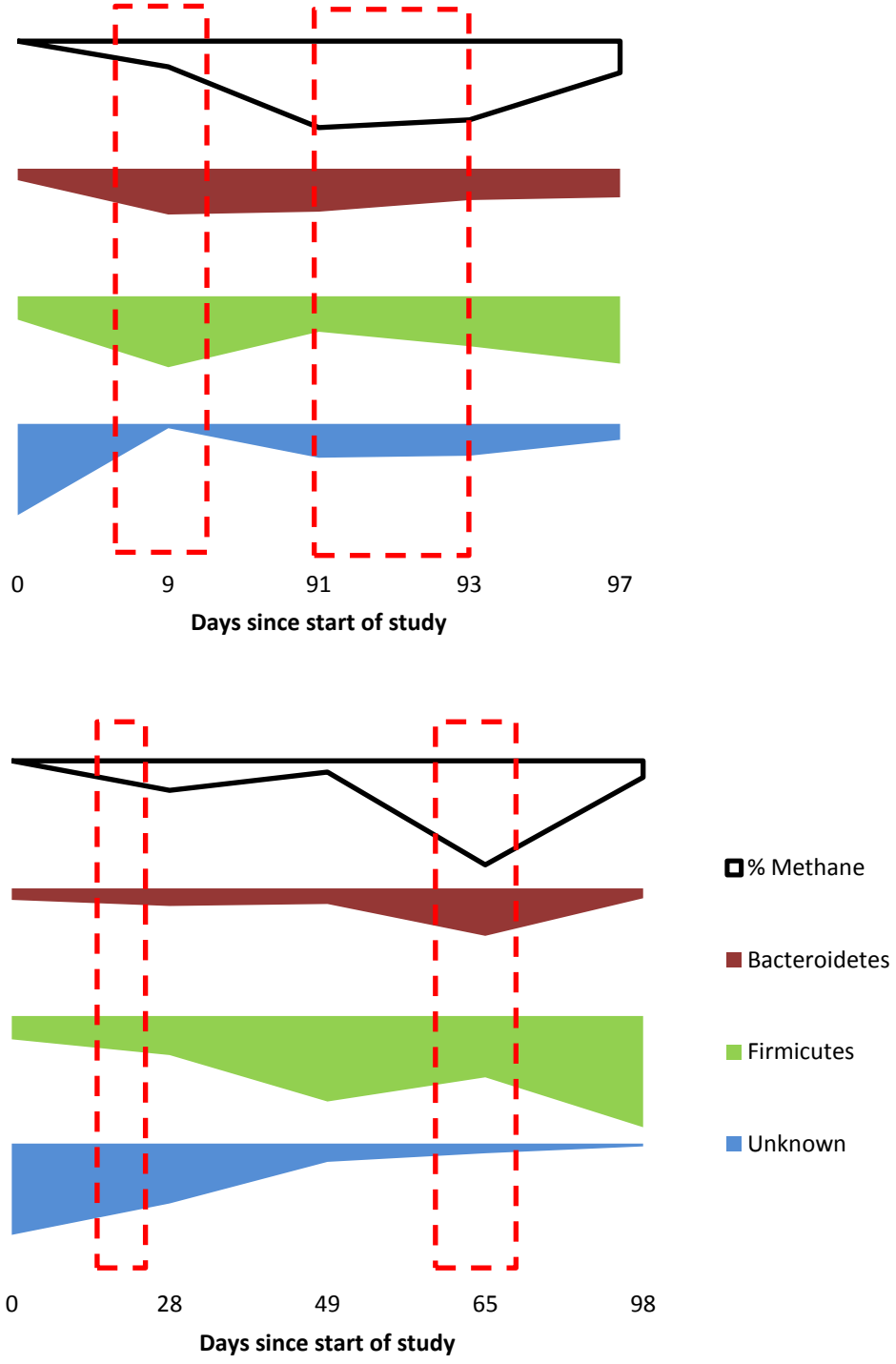

Figure 3.7. Relative changes in bacterial phyla during Multiple OLR increase series using consistent co-digestion substrate digesters 4-6 (top panel) and inconsistent co-digestion substrate digesters 13-15 (bottom panel). Red boxes indicate OLR increase series. x axis is not to scale and only shows days when samples were taken.

Comparison of the bacterial OTUs in digesters 1-9 increase series 2 demonstrated a change in the response to OLR change in digesters pre-exposed to glycerol waste (Figure 3.8; panel a). The pre-exposed digesters at 30 and 50 g l^{-1} glycerol waste from day 97 are placed in a separate cluster (II). This shift immediately precedes the start of the recovery (increase in biogas methane content, and pH from < 6 to > 7; Chapter 2). This indicates that cluster II represents a bacterial community with improved capacity for recovery of inhibited AD. Comparison of the bacterial OTUs in digesters exposed to FOGs waste OLR increase during increase series 2 (digesters 10-15) showed that despite the similarities in lipid fingerprint and AD performance the bacterial OTUs were distinct for the two treatments, with the MDS showing < 75 % similarity between the two treatments (Figure 3.8, panel b).

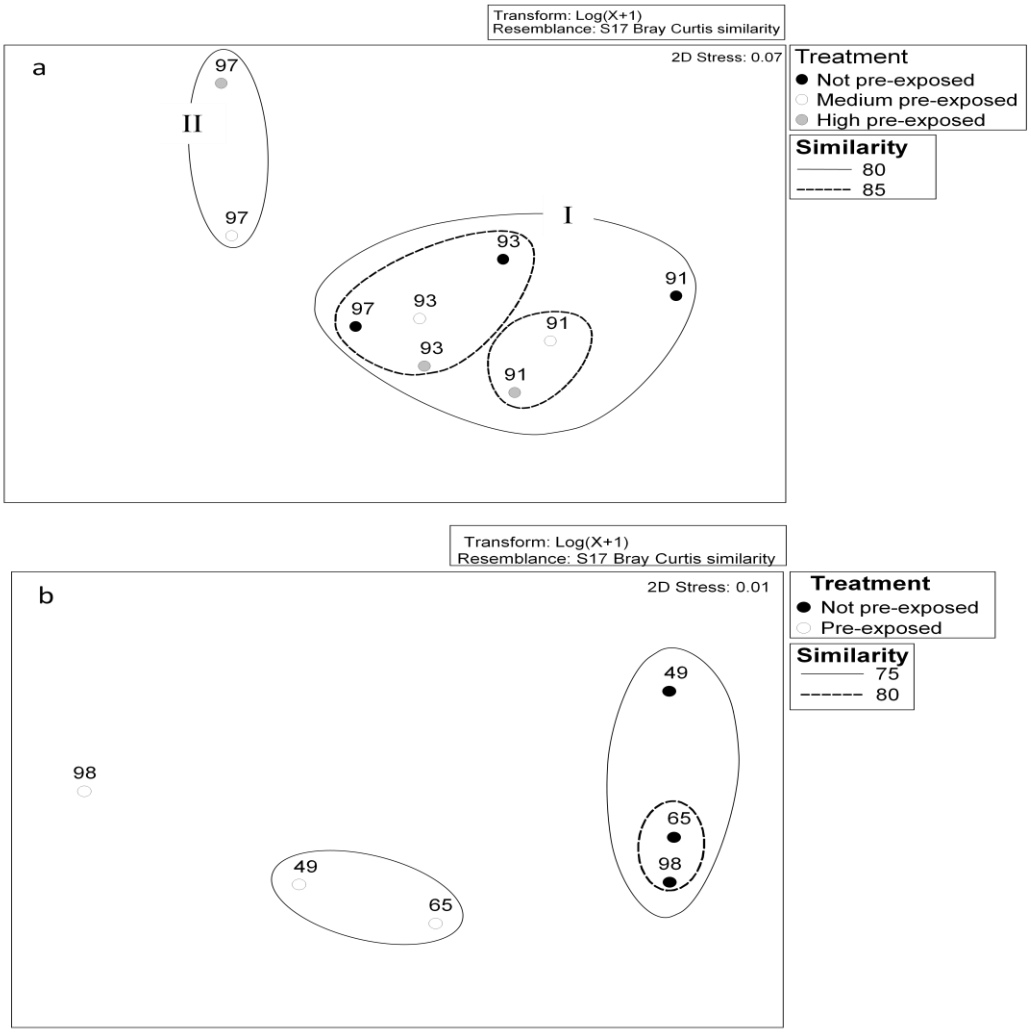

Figure 3.8. Bacterial OTUs structure during OLR increase series 2 in digesters exposed to consistent feedstock 1-9 (a) and inconsistent feedstock 10-15 (b). Profile similarities are based on UPGMA cluster analysis using log transformation data and the Bray Curtis similarity indices. Numbers show days as indicated in Figure 2.1. Black circles show digesters only exposed to OLR increase series 2 and white and grey circles show digesters exposed to OLR increase 1. Triplicate digester averages were used for the cluster analysis.

In the digesters exposed to OLR increase series with glycerol waste only (digesters 1-9) the major differences in OTUs were at family level where members related (or close) to *Clostridia Incertae Sedis* XV were 3 times more abundant in the pre-exposed digesters (6-9) while *Enterococcaceae* were only present and dominant in digesters 1-3 (18 % of OTUs) (Figure 3.9, Figure 3.10, and Figure 3.11). The closest relative to the *Clostridia*

incertae sedis XV OTU was *Cloacibacillus* genus belonging to the *Synergistaceae* family isolated from an AD pilot plant with high butyric acid concentration (FJ799129, 83 %, unpublished). Other *Cloacibacillus* strains have been shown grow a pH 6.5 and carry out acidogenic functions (Looft *et al.* 2013). *Synergistaceae* have been identified in a wide number of habitats including an anaerobic lagoon in a dairy wastewater treatment plant (Baena *et al.* 2000) anaerobic sludge bed reactor treating brewery wastewater (Diaz *et al.* 2007) and goat rumen (McSweeny *et al.* 1993). All cultivable *Synergistaceae* so far isolated have the ability to degrade amino acids into volatile fatty acids and contribute to acidogenesis and acetogenesis via syntrophic relationships with methanogens (Baena *et al.* 1998; Menes and Muxí 2002; Diaz *et al.* 2007; Vartoukian *et al.* 2007). Therefore *Clostridia incertae sedis* XV may play a role in stabilising digesters by improving degradation of VFA and promoting methanogenesis over other pathways. This is consistent with results presented by McMahon *et al.* (2004) who showed that higher numbers syntrophic bacteria in digesters pre-exposed to high OLR was related to improved resilience to organic overload. There were high numbers of the lactic acid producing bacteria (*Enterococcaceae*) and the SRB PLFA marker iso-17:1 in digesters not pre-exposed to glycerol waste. The PLFA iso-17:1 is thought to be related to sulphate reduction during lactate metabolism (Londry *et al.* 2004). In contrast, a link with lactate degradation to methane has been suggested for *Synergistaceae* (Delbès *et al.* 2000; Delbès *et al.* 2001). Therefore the presence of *Synergistaceae* in the pre-exposed digesters may have favoured methane production from lactic acid over sulphate reduction leading to faster recovery. This will be investigated in Chapter 4 as it indicates *Synergistaceae* may be a suitable candidate for bioaugmentation to improved recovery from OLR increase.

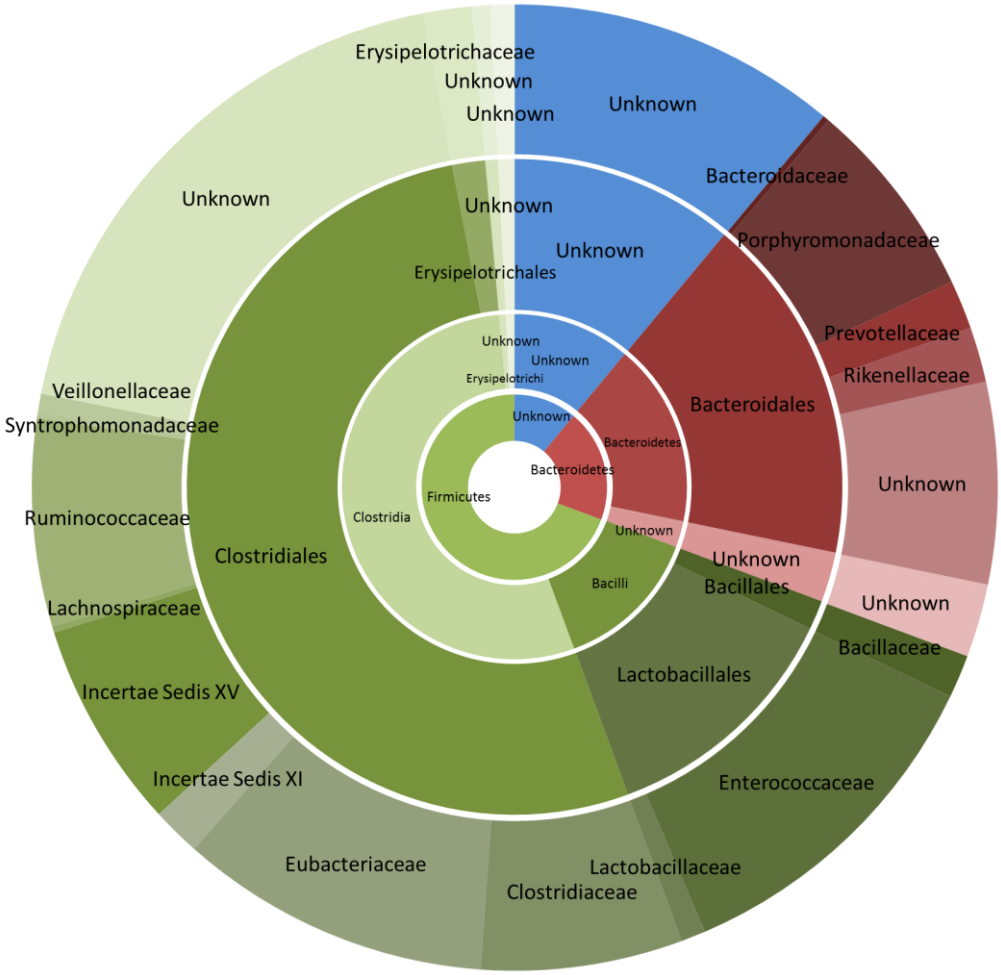

Figure 3.9. Taxonomic affiliations of dominant OTUs (> 5%) during co-digestion of glycerol waste in digesters 1-3, day 97, increase series 2 (6 after start of OLR increase series 2) Rings show Phylum, Class, Order, and Family moving from inner to outer ring**.**

Figure 3.10. Taxonomic affiliations of dominant OTUs (> 5%) during co-digestion of glycerol waste in digesters 4-6, day 97, increase series 2 (6 after start of OLR increase series 2) pre-exposed to glycerol waste at medium OLR digesters. Rings show Phylum, Class, Order, and Family moving from inner to outer ring.

Figure 3.11. Taxonomic affiliations of dominant OTUs (> 5%) during co-digestion of glycerol waste in digesters 7-9, increase series 2, days 97 6 after start of OLR increase series 2) pre-exposed to glycerol waste at high OLR. Rings show Phylum, Class, Order, and Family moving from inner to outer ring.

In digesters 13-15, which were exposed FOGs waste during OLR increase series 2 and glycerol waste in OLR increase series 1, OP10/*Armatimonadetes* members were not detected (as they were in the digesters 10-12 increase series 2 with FOGs) they were instead dominated by the family *Veillonellaceae* (Figure 3.12 and Figure 3.13). The high abundance of *Veillonellaceae* members reflects the increase in the gram negative PLFA 18:1w9cis. The closest cultivated match to this OTU was *Selenomonas ruminantium* (AB198432, 61 %; Sawanon 2011) isolated from sheep rumen. This bacterium was shown to stimulate fibre digestion by the hydrolytic bacteria *Fibrobacter succinogenes* and also to enhance propionic acid production. Thus, *Veillonellaceae* could play a significant syntrophic role in AD at the hydrolysis and acidogenic stages. However, as discussed earlier, these differences in OTUs did not result in different performance between the treatments. This indicates that microbial optimisation such as enhanced resilience to OLR changes and recovery of methane production in AD should be targeted at the acetogenic and methanogenic stages

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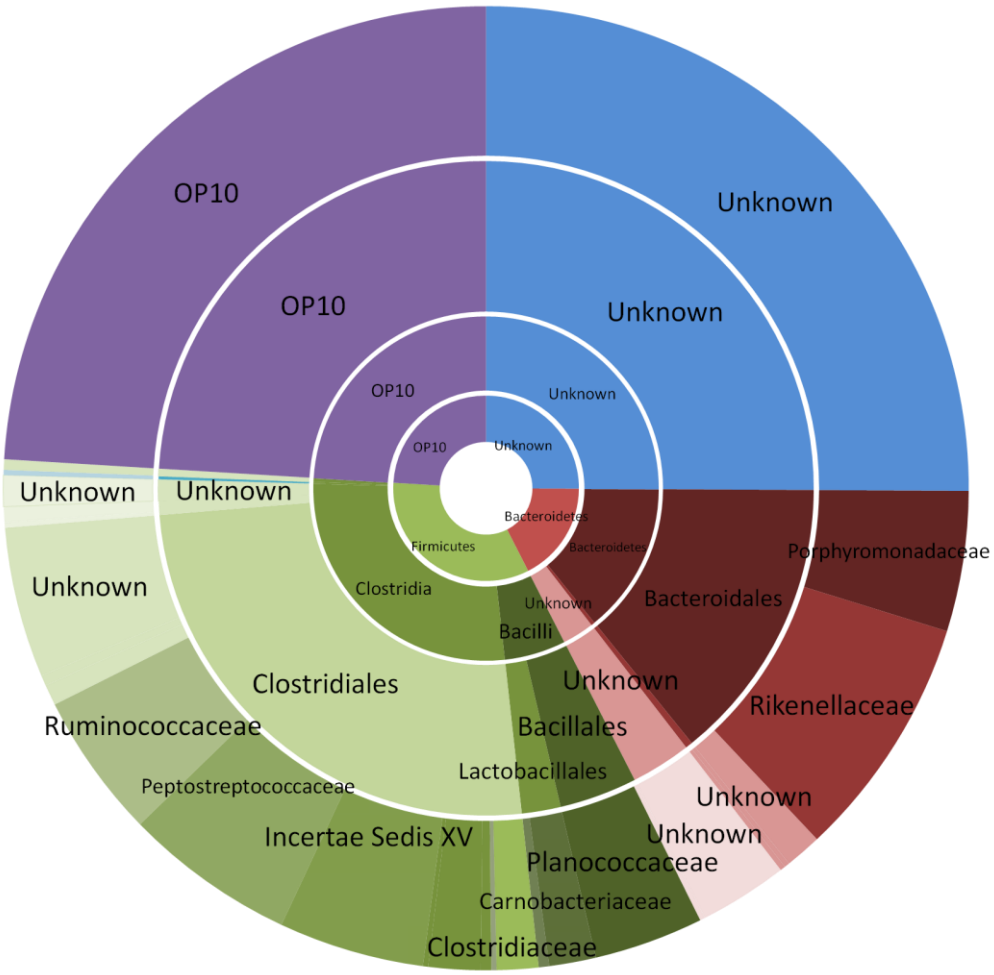

Figure 3.12. Taxonomic affiliations of dominant OTUs (> 5%) during co-digestion of FOGs in digesters 10-13, day 98, increase series 2 (37 days after start of OLR increase series 2). Rings show Phylum, Class, Order, and Family moving from inner to outer ring.

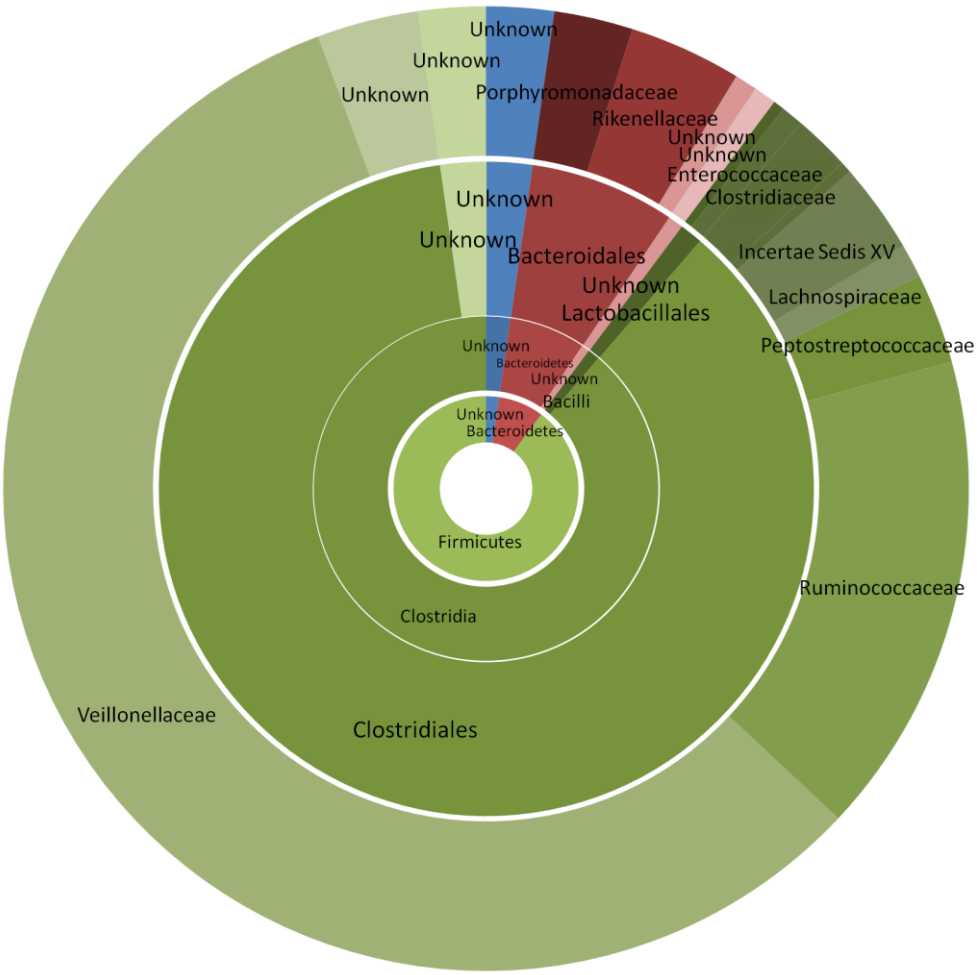

Figure 3.13. Taxonomic affiliations of dominant OTUs (> 5%) during co-digestion of FOGs waste in digesters 13-15, day 98, increase series 2 (37 days after start of OLR increase series) pre-exposed to glycerol waste. Rings show Phylum, Class, Order, and Family moving from inner to outer ring.

Repeated and varied OLR changes using glycerol waste: The effect of a higher OLR during OLR increase series 1 was also investigated (digesters 7 to 9; Table 2.1). A more severe OLR change in increase series1did not result in any differences in the relative proportion of lipid markers and the relationship between bacterial OTUs and biogas methane content (Figure 3.14, Figure 3.15 and Table 3.5).

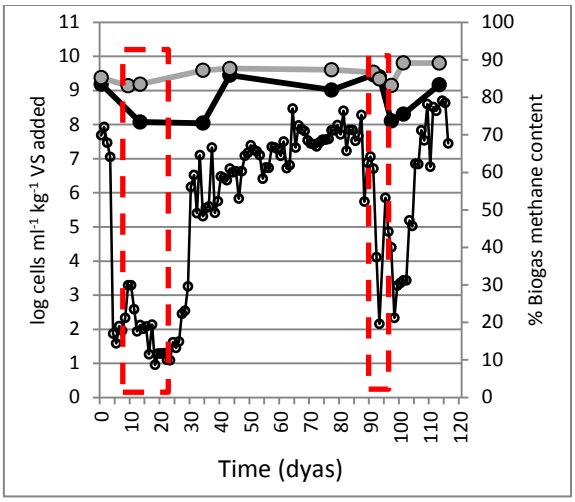

Figure 3.14. Repeated and varied OLR increase series using glycerol waste (digesters 7-9). Percentage methane (white circles) total bacterial biomass (grey circles) and total archaeal biomass (black circles). Blue boxes denote glycerol waste addition increase series as described in Table 1. (n = 3, average standard deviation for % methane = 5, bacterial biomass = 0.1, and archaeal biomass = 0.1). Red boxes indicate OLR increase series.

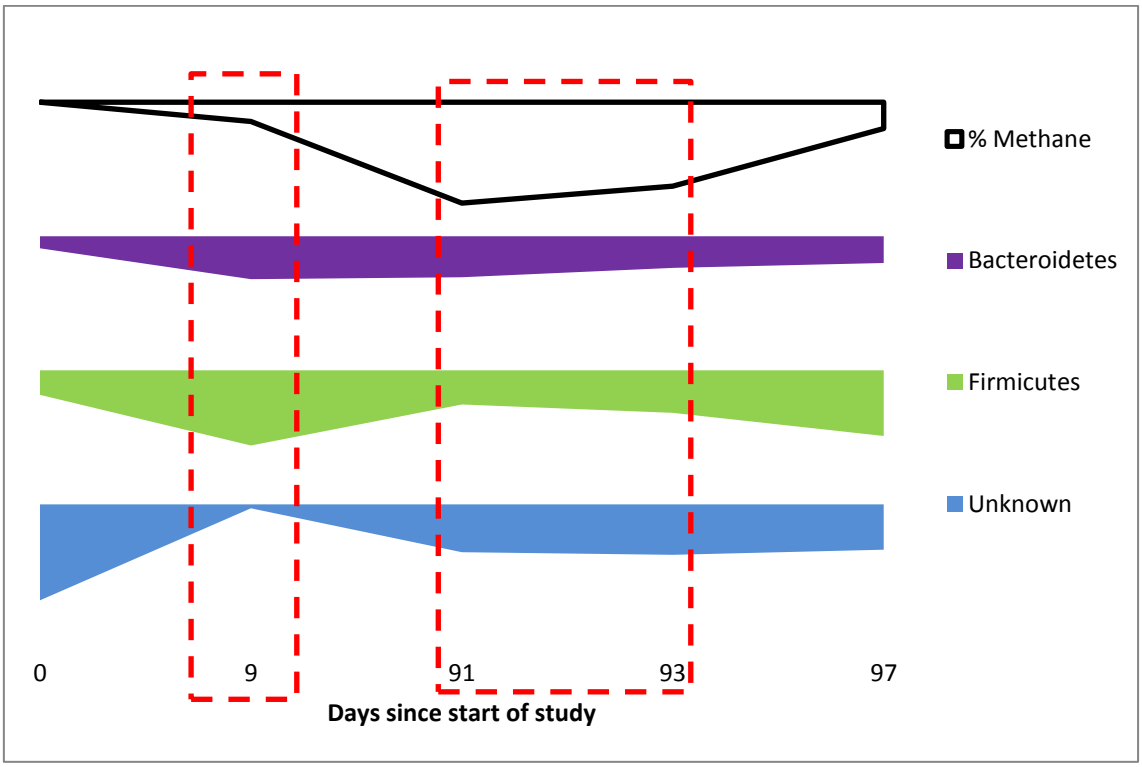

Figure 3.15. Relative changes in bacterial phyla during multiple OLR increase series with varied OLR using glycerol waste in co-digestion with sewage sludge (digesters 7-9). Red boxes indicate OLR increase series. x axis is not to scale.

Table 3.5. Mol % of dominant (> 5 %) PLFA and PLEL in digesters 7-9 during multiple OLR increase series with varied OLR using glycerol waste in co-digestion with sewage sludge Red lines represent OLR increase series.

	Day										
	0	9	13	34	43	77	91	93	97	101	113
PLFA		**increase series 1 high OLR**					**increase series 2 medium OLR**				
iso-15:0	12	12	11	9	9	11	11	10	10	10	11
anteiso-15:0	10	9	11	10	10	11	10	10	10	11	11
16:1 w7cis	14	15	15	18	17	16	16	15	17	17	15
16:0	19	17	17	18	18	16	17	17	17	17	13
iso-17:1	1	1	1	1	1	1	1	1	1	1	1
18:1 w9cis	13	14	11	12	11	12	12	13	13	12	14
18:1w9trans	14	0	4	12	11	11	11	10	10	11	11
18:0	5	5	7	8	9	9	9	8	9	9	10
PLEL											
i20:1	33		44	24	40	29	19	25	15	9	22
i20:0	38		40	0	48	55	67	71	72	14	31
i40:0	29		16	76	12	16	14	3	13	76	47

Triplicate digester average, Average % error = 5.8 %.

3.3.3. Implications for operational monitoring and optimisation of AD

Bioaugmentation improves recovery times during AD: Bioaugmentation with a particular species or consortium of species would allow plant operations to change the existing microbial community so that it is optimised to carry out a specific function (Deflaun and Steffan 2002; El Fantroussi and Agathos 2005). Bioaugmentation to improve AD of LCFA has already been demonstrated by Cavaleiro *et al.* (2010) who showed that bioaugmentation with *Syntrophomonas* resulted in reduced 50 % reduced lag times during start up and gave 30 % higher methane production. The faster recovery of AD after OLR increase discussed in Chapter 2 can be related to changes in bacterial dynamics and increased numbers of specific OTUs as discussed in section 3.3.2. This suggests that it may be possible to use these bacterial groups to bioaugment digesters to stimulate recovery times. *Clostridia incertae sedis* XV (related to *Synergistaceae Cloacibacillus*) was dominant in the glycerol waste pre-exposed digesters (digesters 4 to 15; increase series 1; table 1) and may have played a role in maintaining methanogenesis via syntrophic relationships. As discussed increased numbers of syntrophic bacteria has been related to improved resilience of AD by other authors and therefore should be considered for bioaugmentation.

Using changes in digester conditions to optimise AD: An alternative to seeding a species directly into an AD plant would be to manipulate process conditions to promote a community more resilient to changes in OLR and co-digestion substrate. Flexibility and functional diversity which is a key factor in improved AD performance and stability, can be promoted in AD by designs that incorporate spatial heterogeneity such as, baffled digesters, granular substrates, and membrane reactors (Fernández *et al.* 2000;

Hashsham *et al.* 2000; Briones and Raskin 2003). Briones *et al.* (2003) suggested incorporating heterogeneity temporally (such as changes in OLR of feedstock) as well as spatially could be used to optimise the AD microbial community. Clearly the results presented in this chapter reinforce these suggestions. However further research is still required in this area as, while there is mounting evidence that substrate pulses and OLR variation can optimise the composition, dynamics, and physiology of the microbial community in AD, variation in feedstock resulted in worse performance (Chapter 2) additionally it is still not fully understood how to best implement this at full scale (McMahon *et al.* 2004; Palatsi *et al.* 2009; Palatsi *et al.* 2010).

Using microbial community for process monitoring in AD: Maintaining stability in AD is a fine balance as the operator must ensure that the OLR is balanced with the production of VFA and full degradation of the available volatile solids is possible with the shortest possible hydraulic retention time (HRT). As feedstock availability fluctuates operators need to know how to adapt feedstock change co-digestion substrates without causing AD instability. A major component of this is an understanding of what parameters to monitor. The archaeal biomass (PLEL) was always correlated with biogas methane content for both co-digestion substrates at $p < 0.001$ (Table 3.6 and Figure 3.9). In contrast no clear relationship between the bacterial biomass (PLFA) and biogas methane content was observed especially when FOGs waste was being co-digested (Table 3.6). These results demonstrate than archaeal biomass is a key parameter in biogas quality, however, this is unsurprising as Archaea are the sole producers of methane in AD (Garcia *et al.* 2000; Demirel and Scherer 2008). Our results also show that digesters with biogas production higher than 0.2 m^3

kg^{-1} VS day^{-1} had an average archaeal biomass of 9.1 ± 0.4 log cells ml^{-1} kg^{-1} VS added, in contrast digesters with biogas production < 0.2 had average archaeal biomass of 8.3 ± 0.4 log cells ml^{-1} kg^{-1} VS added. This establishes a relationship between overall biogas production and archaeal biomass which is not an obvious outcome as digesters could switch to other fermentation pathways resulting in the production of CO_2 and or H_2S to maintain biogas production with low archaeal biomass. The implication of this result is that the syntrophic interactions between archaea and bacteria are beneficial to both groups not just the archaea and that high archaeal biomass improves production as well as quality of biogas.

Table 3.6. Summary of linear regressions of biogas methane content and biomass under varying conditions.

Co-digestion substrate	Archaea/Bacteria	P value	Slope	R^2
All	Archaea	<0.001	0.02	0.61
	Bacteria	0.007	0.001	0.1
Glycerol waste	Archaea	<0.001	0.002	0.42
	Bacteria	<0.006	0.005	0.26
FOGs waste	Archaea	<0.001	0.002	0.85
	Bacteria	0.2	NA	NA

As sequencing technology becomes cheaper it may become practical for AD operators to monitor the structure of the microbial community (Bjerketorp *et al.* 2008; Talbot *et al.* 2008; Franke-Whittle *et al.* 2009). Increased numbers of Clostridia was consistently associated with poor AD performance in this study. Therefore this group could also be a candidate for AD monitoring. Clostridia are an important group of anaerobic bacteria in

AD that can carry out key stages of AD, fermentation, homoacetogenesis and syntrophic acetate and propionate oxidation (Delbès *et al.* 2001; Park *et al.* 2001; Gerardi 2003; McMahon *et al.* 2004; Gerardi 2006).

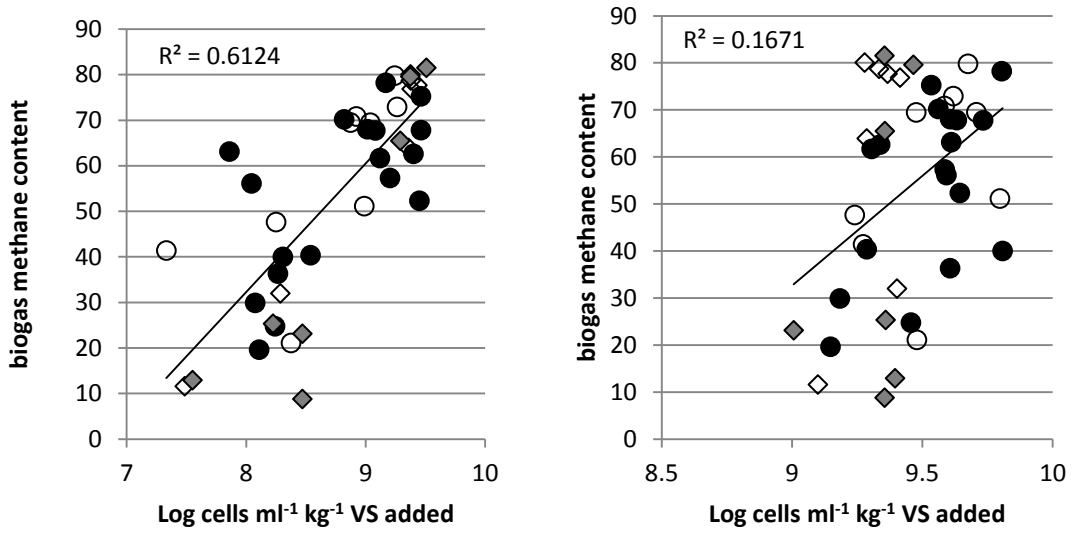

Figure 3.16. Scatter plot of archaeal biomass (left panel) and bacterial biomass (right b) against biogas methane content in all conditions tested. Black circles = repeated glycerol waste addition (increase series 1+2, digesters 4-9) white circles: glycerol waste (increase series 2 only, digesters 1-3) grey diamonds: glycerol waste increase series 1 and FOGs waste increase series 2 (digesters 13-15) white diamonds: FOGs waste only (increase series 2, digesters 10-12). The solid line represents the linear regressions both are significant at $p < 0.01$ and with R^2 of 0.6 for archaea and 0.2 for bacteria.

It is probable that after OLR increase, clostridia, as a generalist group capable of a number of metabolic pathways were able to take advantage of the increased organic loading and dominate the community. In Chapter 2 a number of performance indicators for AD were assessed, using the results in this chapter we can add bacterial biomass in terms of PLFA and PLEL and Clostridia to the schematic proposed in Chapter 2 (Figure 3.17). Further analysis is required to confirm values for these parameters and to further

investigate specific if specific families or OTUs can be related to performance, this is presented in Chapter 5.

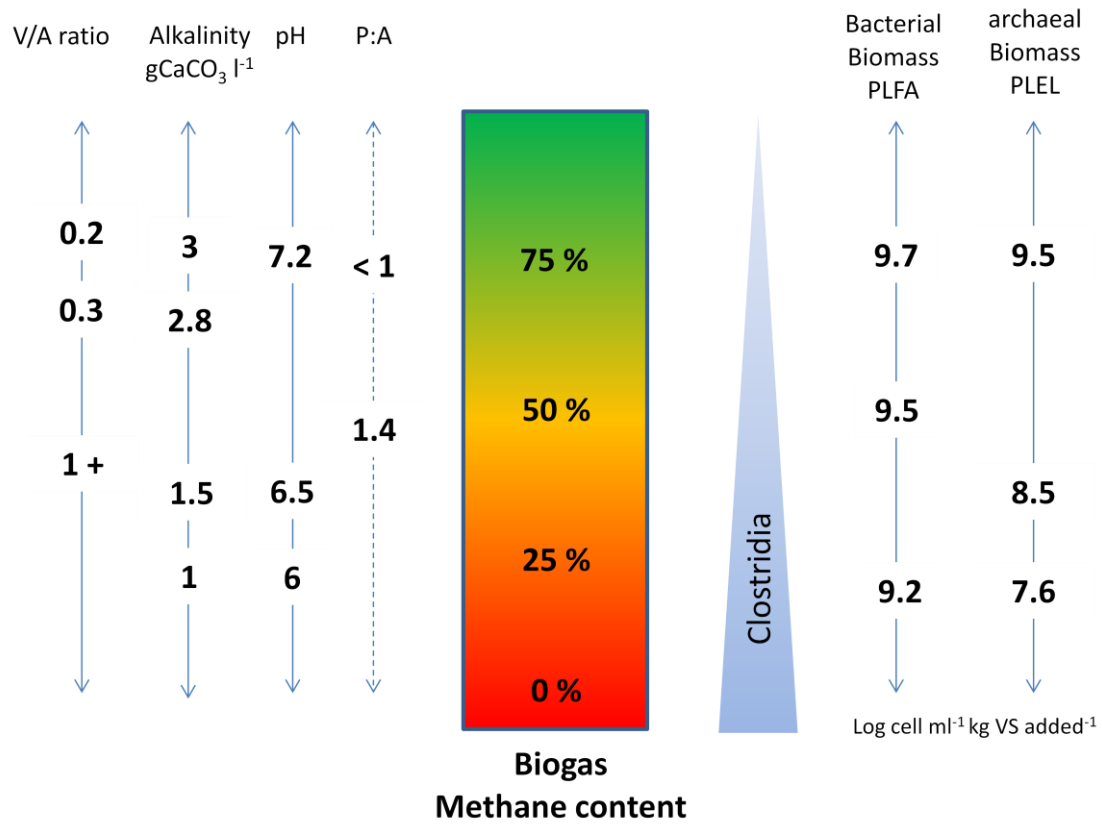

Figure 3.17. Summary of the critical parameters and microbial community in terms of performance (biogas methane content) based on our results. Expanded from Figure 2.9.

3.4. Conclusions

OLR increase series using sludge co-digestion with glycerol waste and/or FOGs addition induced changes in the microbial community structure, abundance and dynamics. The most noticeable changes were a 5 and 15-fold decrease in bacterial and archaeal biomass. The dominant bacterial OTUs also varied for the two co-digestion substrates, There was a high proportion of candidate phylum OP10 (26 %) in digesters co-digesting FOGs waste, in contrast the digesters co-digesting glycerol waste had

higher proportions of *Clostridia Incertae Sedis* XV (38 %) and *Ruminococcaceae* (17 %). Further to this, *Firmicutes* members doubled in both treatments when biogas methane content decreased to < 30 %.

Despite these differences, similar response trend to OLR increase series was observed among digesters, which was a decrease of the archaeal biomass associated with a decrease of gas production and methane content and increase in *Clostridia*. These changes were also seen when digesters were exposed to multiple changes in OLR with glycerol waste and FOGs waste confirming that these parameters could form the basis of microbial monitoring of AD.

Digesters that were exposed to multiple OLR increase series recovered biogas methane content faster when exposed to further OLR increase with the same feedstock (Chapter 2). Archaeal biomass decrease was delayed by 3 days in digesters exposed to multiple OLR increase series with glycerol waste, indicating increased resilience to OLR increase in the archaea. There was also no change in the Mol % of 18:1w9trans PLFA (which had previously decreased after OLR increase) indicating adaptation or structure change in the *Clostridia* or *Bacilli*. The OTUs analysis showed that there were higher numbers of *Clostridia incertae sedis* XV (closest cultivated match *Cloacibacillus* genus, *Synergistaceae* family) in the pre-exposed digesters which could have played a role in stabilising digesters through syntrophic interactions with Archaea. This indicates that recovery times of upset digesters could be improved via bioaugmentation or manipulations of performance to increase numbers of this bacterial family.

Chapter 4: The effect of feedstock and OLR change on volatile fatty acids (VFA) production and microbial community dynamics in anaerobic digestion.

Abstract: FOGs waste or glycerol waste was used to increase OLR in laboratory scale anaerobic digesters fed with sewage sludge. OLR increase using FOGs generated two different stages of VFA production. An initial stage characterised by accumulation of acetic acid and high biogas production followed by a period of accumulation of propionic acid and poor biogas production. Whereas OLR change using glycerol waste produced lactic acid as the main VFA component followed by parallel production of acetic and propionic acid. However, when digesters were exposed to repeated changes in OLR with glycerol waste the production of VFA was different indicating a shift in the metabolic pathways. The microbial community did not produce lactic acid and was able to uphold the conversion of propionic acid into acetic acid. This resulted in faster recovery as discussed in Chapter 2 and shows that the changes in microbial community structure observed in Chapter 3 could be linked to changes in digester function. The analysis of the results allowed to established links between the bacterial families *Rumminococcaceae, Entrococcaceae, Porphymonadaceae, Prevotellaceae,* with the iso bacterial lipid markers and the production of lactic, propionic and iso butyric acid. Conversely the bacterial family *Clostridia incertae sedis* XV and the archaeal lipid marker i20:0 were linked to acetic acid concentration. This confirms the outcome of chapter 3 which relates high OTUs numbers of *Clostridia incertae sedis* XV/*Synergistaceae* to improved conversion of lactic, propionic and n-butyric acids into acetic acid and ultimately methane via syntrophic relationships with archaea, resulting in faster recovery of biogas methane content after OLR change.

4.1. Introduction

The effects of repeated changes in organic loading rate (OLR) on the performance of anaerobic digesters have been investigated in Chapter 2 and 3. It was shown that digesters exposed to repeated OLR increase series with glycerol waste were able to recover 1.5 hydraulic retention times faster and had improved performance in terms of methane yields compared to digesters exposed to a single OLR increase series. In contrast digesters subjected to OLR increase series using two different co-digestion substrates did not show any improvement in performance. The aim of this chapter is to further investigate the influence of co-digestion feedstock on the production and composition of volatile fatty acids (VFA) produced by the bacterial community and to link this to the performance and the structure of the microbial community.

During AD the bacterial community hydrolyses complex organic molecules (hydrolysis) which are converted into organic acids (acidogenesis) the organic acids are then converted into acetate (acetogenesis) which is the main precursor for methane production by Archaea (Conrad 2005; Liu and Whitman 2008). If the acetic acid produced by the bacterial community cannot be rapidly processed by the archaeal community it will accumulate, resulting in lower pH and processing of the acid through other pathways such as hydrogen production or sulphate reduction (Van Den Berg *et al.* 1980; Conrad 1999; Schnürer *et al.* 1999; Zhang *et al.* 2007; Qu *et al.* 2009; Wang *et al.* 2009b; Laukenmann *et al.* 2011). It has been evidenced that high concentrations of acetic acid in itself are not inhibitory to the anaerobic digestion process, as good methane production was observed in presence of concentrations of acetic acid >1600 mg l^{-1} by several authors (Delbès *et al.* 2001; Wang *et al.* 2009b; Ros *et al.* 2013). When acetotrophic methanogenesis is inhibited or saturated accumulation of propionic

acid and other VFA can occur. However, similarly to acetic acid, propionic acid accumulation is a symptom of digester imbalance rather than a cause of inhibition. Studies have shown that when pH is controlled, methanogenesis is not inhibited by concentrations of propionic acid as high as 6 g l^{-1} (Gourdon and Vermande 1987; Ahring *et al.* 1995; Wang *et al.* 2009b). Therefore it is possible that understanding what factors influence the production of VFA in AD may improve our ability to predict and control the process.

Previous studies have shown that VFA concentrations play an important role in structuring the archaeal community in AD (Griffin *et al.* 1998; Delbès *et al.* 2001; Karakashev *et al.* 2005; Hori *et al.* 2006). Ros *et al.* (2013) showed that gene copy numbers of *Methanosarcina* increased by 3 orders of magnitude when the concentration of acetic acid doubled to > 1600 mg l^{-1} and Ripley ratio increased from 0.3 to > 0.5. A shift to *Methanosarcina* when VFA start to accumulate in digesters, with *Methanosaeta* dominating at < 1 g l^{-1} VFA, is also reported by other authors (Griffin *et al.* 1998; Karakashev *et al.* 2005). Despite the great deal of research on the relationships between VFA composition and their influence on the archaeal community dynamics, our understanding of the links between the bacterial community dynamics and the VFA composition and concentration is still limited. A link between high VFA concentration and increase in Clostridia was proposed by Delbès *et al* (2001) whereas, Hori *et al* (2006) suggested that pH had a greater impact on the structure of the bacteria with VFA accumulation effecting the archeal community, causing a 10,000 fold increase in gene copy numbers for *Methanothermobacter*. The application of next generation sequencing (NGS) represents an opportunity to gain a deeper understanding of the microbial communities in AD, as demonstrated by Werner *et al* (2012) and Schlüter *et al* (2008)

who analysed the bacterial communities of nine full-scale AD plants over a yearlong monthly time series and the metagenome of a biogas-producing microbial communities respectively. In these studies the deep resolution available with NGS gave insights into the structure and function of AD microbial communities under varying conditions that were previously difficult to identify due to high functional redundancy in bacterial populations of AD as shown by Fernández *et al.* (1999).

The aim of this chapter is to further investigate the relationship between VFA production, AD performance and the microbial community. In chapter 2 variations in the ratio between propionic and acetic acid during OLR increase with different co-digestion substrates indicated that variation in the VFA profile was linked to performance but that these links were not as clear as previously suggested. Further investigation was needed on the microbial dynamics and the VFA production and composition to fully understand the importance of these parameters. To do this a combined approach of 454-Pyrosequensing and lipid fingerprinting was used to study the links between the organic acids accumulated during OLR changes and the microbial community.

4.2. Methods

4.2.1. Operational parameters

Operational conditions are given in section 2.2.1.

4.2.2. Biogas production, methane concentration and physicochemical characterisation

Gas production was measured daily by water displacement in a glass column (150 x 5 cm, green food dye was used to aid measurement). Gas volume was corrected to standard ambient temperature and pressure (SATP) (25 °C and an absolute pressure of 100 kPa). Methane content was measured using a SERVOPRO1400 CH_4 gas analyser (Servomex, UK) according to manufacturer recommendations. pH and alkalinity were measured according to standard APHA methods (APHA 1989). V/A ratio was calculated as the ratio between total VFA (measured by HPLC analysis) and total alkalinity measured according to standard APHA methods. Propionate : Acetate (P:A) ratio was calculated as the ratio between propionic and acetic acid concentration measured by HPLC analysis, a ratio of greater than 1.4 is considered an indicator of imminent digester failure (Hill *et al.* 1987).

4.2.3. Volatile fatty acids analysis

A 40 ml aliquot of the digestate was centrifuged at 5000 g for 5 min and the supernatant was filtered to < 0.45 μm with a syringe filter. 5 μl of 97 % sulphuric acid was added (to avoid acid degradation when stored) and the sample was stored at – 20 º C until analysis. 100 μl of the sample was injected into a HPLC (535 Kontron, Bio-TEK, UK) equipped with a Bio-Rad fermentation column (Cat 125-0115) 300 x 7.8 mm maintained at 65ºC, and a UV detector at 210 nm. The mobile phase was 0.001 M

sulphuric acid in HPLC grade water with a flow rate of 0.8 ml/min. Acetic, propionic, n-butyric, iso-butyric and lactic acids were quantified using an external multilevel calibration ranging from 0.1 g l^{-1} to 5 g l^{-1}. The % error in the repeatability of measurements for each acid was 0.6, 0.77, 0.72, 1.13, and 3.35 % respectively.

4.2.4. Phospholipids and ether-linked isoprenoids analysis.

Detailed methods for the Phospholipids and ether-linked isoprenoids analysis are given in chapter 3.

4.2.5. Taxonomic affiliation of PLFA and PLEL

The taxonomic affiliations are summarised in Table 4.1. Gram-positive bacteria were represented by the series of iso and anteiso branched saturated PLFA. Gram-negative bacteria were represented by cyclopropane, hydroxyl and monounsaturated PLFA. The 16:0 straight chain PLFA has been previously demonstrated as an ubiquitous bacterial marker (Piotrowska-Seget and Mrozik 2003). The PLFA 18:2w9cis and 18:1w7trans used as markers for clostridia. The PLEL i20:0 was used as a marker for the E*uryarchaeota*, i20:1 as a marker of the aceticlastic methanogens belonging to *Methanosarcina* and i40:0 as a marker for hydrogenotrophic methanogens belonging to *Methanobacterium, Methanococcus, Methanopyrus, and Methanothermus* (Gattinger *et al.* 2002; Radl *et al.* 2007).

Table 4.1. Summary of PLFA and PLEL taxonomic affiliation (adapted from Gattinger *et al.* 2002; Gattinger *et al.* 2003; Londry *et al.* 2004; Oravecz *et al.* 2004; Radl *et al.* 2007).

		Taxonomic affiliation
Straight saturated		
11:0		
12:0		
13:0	ubiquitous	*δ-Proteobacteria*
14:0		*Actinobacteria/ δ-Proteobacteria*/ low GC G+
15:0		*Actinobacteria*
16:0		*Actinobacteria*/ low GC G+/CFB/ *δ-Proteobacteria*
17:0		Δ-*Proteobacteria*
18:0		low GC G+ *Bacillus/ Clostridium*
20:0		
iso and anteiso branched		
iso-15:0	G+	low GC G+/*Bacillus/ Actinobacteria*
anteiso-15:0		*Actinobacteria*/ *Bacillus/* low GC G+/CFB[a]/ δ-*Proteobacteria*
iso-16:0		low GC G+/ *Bacillus/ Actinobacteria*
iso-17:1		CFB (*Cytophaga*)/SRB[b]
cyclopropane		
cyc 17:0	G-	Anaerobes/ *Bacillus/ Clostridium* / δ-P*roteobacteria*
cyc-19:0		Anaerobes/ *Bacillus/ Clostridium* / δ-*Proteobacteria*
mono-unsaturated		
16:1 w7cis	G-	*Bacillus/ Clostridium*
18:2 w6cis		*Actinobacteria* / *Bacillus/ Clostridium*
18:1 w9cis		Anaerobes/ *Bacillus/ Clostridium*
18:1w9trans		Anaerobes/ *Bacillus/ Clostridium*
poly-unsaturated		
18:2w6,9		Fungi
hydroxy		
2OH-10:0	G-	*δ-Proteobacteria*
2OH-12:0		*δ-Proteobacteria*
3OH-12:0		*δ-Proteobacteria*
3OH-14:0		*δ-Proteobacteria*
2-OH 16:0		*δ-Proteobacteria*/CFB
isoprenoids		
i20:0	Methanogens	*Euryarchaeota*
i20:1		*Methanosarcina*
i40:0		Hydrogenotrophic methanogens

[a] CFB: *Cytophaga-Flavobacteria-Bacteroides* [b]SRB: sulphate-reducing bacteria

4.2.6. 454-pyrosequencing analysis and Bioinformatics

Detailed methods for 454-pyrosequencing analysis and Bioinformatics are given in Chapter 3.

4.2.7. Statistical analysis

Cluster analysis and nMDS was carried out in r (http://www.R-project.org/) using the package "Vegan" after Oksanen (2013). The concentration of VFA was used to calculate Bray Curtis indices using the function "vegdist" and then the function "hclust" was used for cluster analysis using UPGMA. The function "cutree" was then used to group similar samples based on the cluster analysis. Non-metric multi-dimensional scaling (nMDS) ordination was carried out using the function "metaMDS". Vectors for bacterial OTUs and PLFA and PLEL lipid markers were then fitted to the nMDS using the function "envfit", only those that were significant at $p = 0.05$ were plotted on the nMDS.

4.3. Results and discussion

4.3.1. Effect of single OLR increase series on VFA production using glycerol waste or FOGs waste as a co-digestion substrate

Digesters were exposed to a single OLR increase series with glycerol waste (digesters 1-3) or FOGs waste (digesters 10-12) as a co-digestion substrate at an OLR of 2.9 kg VS m^{-3} d^{-1}. Lactic acid was the major fermentation product in the initial stages of glycerol waste co-digestion (53 % of total acids) with concentrations of 5 ± 0.6 g l^{-1} (Figure 4.1). There was also a high concentration of iso butyric acid which reached 2.1 ± 0.5 g l^{-1} on day 97, suggesting that production of these two acids may be linked. After day 97, acetic and propionic acids were the dominant organic acids and were produced in parallel. In contrast to glycerol waste co-digestion, production of VFA during FOGs waste co-digestion was sequential (rather than parallel) and can be split into two stages (Figure 4.2). During the first stage acetic acid was the dominant organic acid produced, comprising > 70 % of the VFA fingerprint and reaching a maximum of 6.5 ± 0.6 g l^{-1} on day 65. After day 75(second stage) propionic acid was the dominant VFA (5.99 ± 1.01). The shift from acetic acid production to propionic acid production indicates a shift in the metabolic function of the microbial community, a schematic of the two VFA production periods is presented in (Figure 4.3).

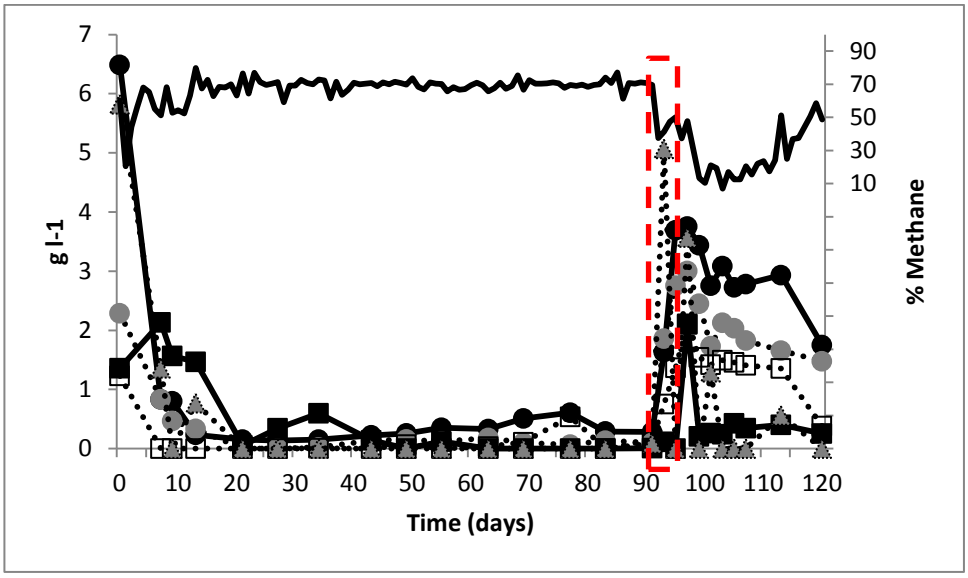

Figure 4.1. Concentrations of VFA in digesters 1-3, red box indicates OLR increase series 2 (day 91). acetic acid (black circles and solid lines) propionic acid (black circles and dashed lines) iso butyric acid (black squares, solid lines) n butyric acid (clear squares, dashed lines) and lactic acid (grey triangles, dashed lines). Solid line shows biogas methane content. Triplicate digester average, error bars are not shown to improve legibility. average standard deviation for acetic acid = 0.3, propionic acid = 0.2, iso butyric = 0.01, n butyric = 0.1, lactic = 0.1, and % methane = 4.8.

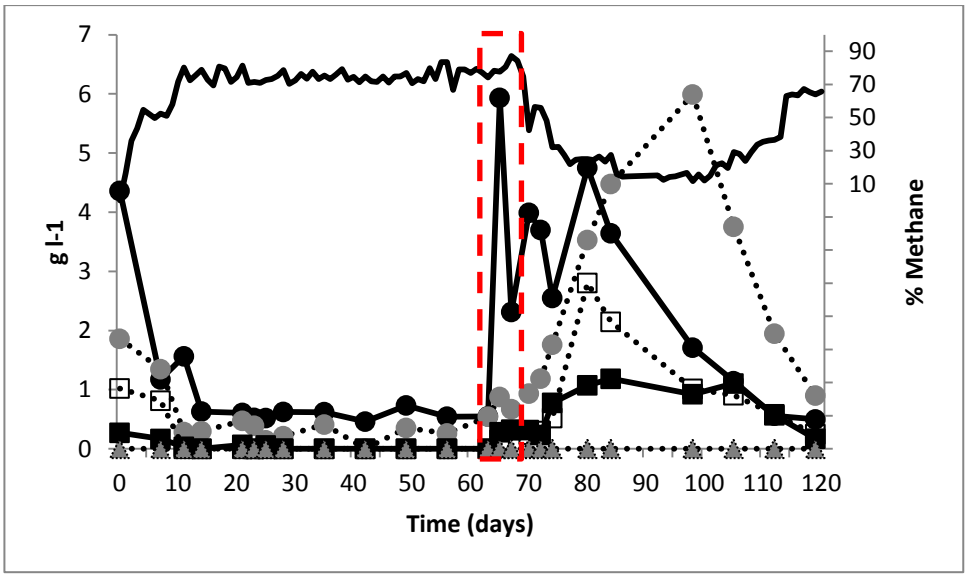

Figure 4.2. Concentrations of VFA in digesters 10-12, Red box indicates OLR increase series 2 (day 63), acetic acid (black circles and solid lines) propionic acid (black circles and dashed lines) iso butyric acid (black squares, solid lines) n butyric acid (clear squares, dashed lines) and lactic acid (grey triangles, dashed lines). Triplicate digester average, error bars are not shown to improve legibility. average standard deviation for acetic acid = 0.4, propionic acid = 0.5, iso butyric = 0.1, n butyric = 0.2, lactic = 0, and % methane = 4.2.

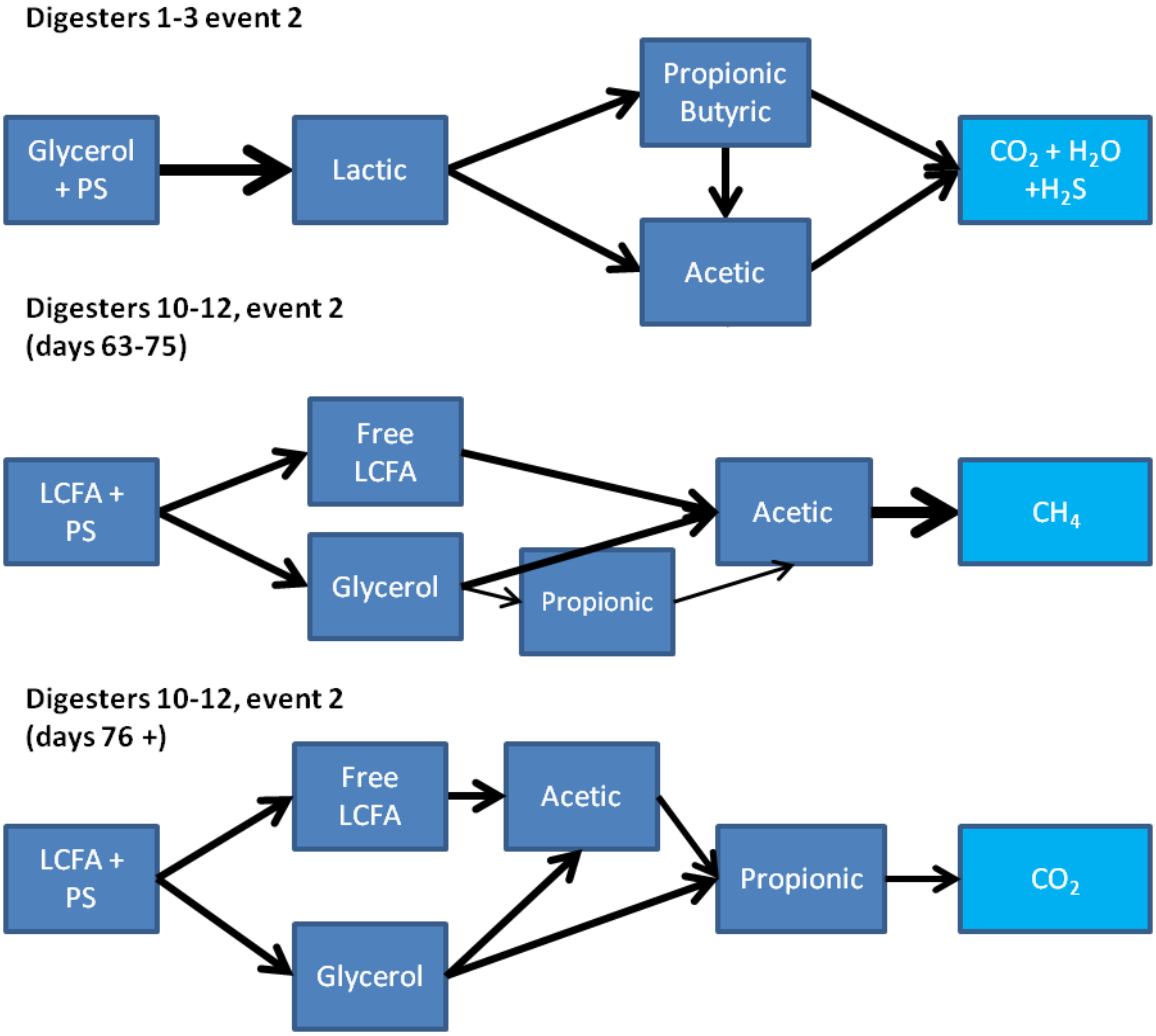

Figure 4.3. Schematic of possible pathways for digestion of glycerol waste and FOGs based on VFA profiles during increase series 2.

The major pathway for the microbial degradation of lactic acid is its conversion to propionic acid (Ren *et al.* 1997; Zhang *et al.* 2007). This is consistent with the VFA profiles in digesters 1-3 as high concentrations of propionic acid increase when lactic acid concentration decreases (>2.5 g l^{-1}) (Figure 4.1). It is well established that accumulation of propionic acid is indicative of digester imbalance in the methanogenic process and therefore high concentration of lactic acid after addition of glycerol waste to feed is also undesirable for methane production. A schematic of the suggested

degradation pathways for glycerol waste based on our results and literature data those of Zhao *et al.* (2008) and Dinkel *et al.* (2010) is presented in (Figure 4.3). An acetic acid concentration of 4 g l^{-1} was proposed as a max value for optimal methane production (Stafford 1982; Wang *et al.* 2009b). However, after addition of FOGs waste to digesters 10-12, methane content higher than 80 % (chapter 2) was observed with acetic acid concentrations above 4 g l^{-1}. Indeed, although V/A ratio increased after addition of FOGs waste (chapter 2), the reactors contained sufficient buffering capacity to keep the pH above 7.3 ± 0.2 (chapter 2). This demonstrates that if drop in pH can be mitigated, high concentrations of acetic acid result in methane production being favoured over other metabolic pathways. Similar findings were presented by Ros *et al.* (2013) who showed that biogas methane content remained above 70 %, with acetic acid concentrations ranging from 1-2.6 g l^{-1}, when pH was maintained between 7 and 7.5. These results further support the hypothesis that pH is the key factor in maintaining high biogas methane content and that an increase in the ratio of VFA to alkalinity or V/A ratio, which precedes changes in pH, is a key monitoring parameter for AD.

4.3.2. Effect of repeated OLR increase series on VFA production using one or two co-digestion substrates

In chapter 2, analysis of the performance parameters of AD showed that digesters pre-exposed to glycerol waste recovered 1.5 HRT faster than the control when exposed to glycerol waste again. Analysis of the P:A ratio indicated that changes in VFA production could be related to this improvement but that a deeper analysis of the VFA profile was required to understand this relationship.

Digesters receiving glycerol waste in the feed for the first time (digesters 4-6, increase series 1 and digesters 1-3, increase series 2) showed very similar behaviour. Specifically there was an initial high production of lactic and iso butyric acid later substituted by a parallel production of propionic and acetic acid (Figure 4.4). After the second OLR increase series (increase series 2, digesters 4-6) the production of VFA was markedly different, showing that the processing of feedstock had changed in addition to (as shown in Chapter 3) the shifts in microbial community structure. Lactic acid was less than 6 % of the total organic acid fingerprint, in contrast to > 50 % after the first OLR (Figure 4.4). Accumulation of acetic and propionic acids occurred in parallel between day 91 and 105. After day 105 the production of these two acids occurred sequentially. This is in contrast with the results obtained for the digesters that were only exposed to one OLR change, where acetic and propionic acids accumulated in parallel throughout the trial. It is possible that after day 105, the propionic acid is converted into acetic acid resulting in faster recovery, as described in chapter 2. The concentration of iso-butyric acid, thought to be associated to high concentrations of lactic acid, was never above 1 g l^{-1}, about 50 % lower than digesters only exposed to one OLR change. Therefore a lower accumulation of iso butyric acid was also linked to faster recovery. This demonstrates that after OLR change the metabolic function of the microbial community, in addition to the community resilience structure, is responsible for improved performances. A schematic for proposed pathway is presented in Figure 4.6.

After the second OLR change, when FOGs waste was used as co-digestant (digesters 13-15, OLR increase series 2) the digesters pre-exposed to glycerol waste processed VFA in the same way as digesters 10-12 not pre-exposed to glycerol waste, Figure 4.5. These results suggest that multiple OLR increase series using different feedstocks were

not able to produce any change in the microbial metabolic pathways, which is consistent with the operational performances of the digesters observed in Chapter 2. However the microbial communities of these digesters were shown to be distinct in Chapter 3, demonstrating high functional redundancy in these microbial communities. These results highlights the value of understanding both microbial community structure and function in AD as a change in one may not always result in change of the other (Fernández *et al.* 1999; Fernández *et al.* 2000; Zumstein *et al.* 2000; Wang *et al.* 2010b; Wang *et al.* 2011). High functional redundancy could represent a barrier to microbial optimisation of AD. However research has shown that (including chapter 3) consistent links between performance and microbial community structure can be found in AD, particularly in the more specialist and less dominant groups of bacteria/archaea which show more correlation with physicochemical parameters (Briones and Raskin 2003; Rittmann 2006; McMahon *et al.* 2007; Werner *et al.* 2012). Therefore a deeper understanding of the microbial community using a combined approach of techniques such as lipid profiling, that can show changes in physiology of bacteria, and 454-pyroseasing, which can resolve the rare diversity, are key tools in microbial optimisation of AD.

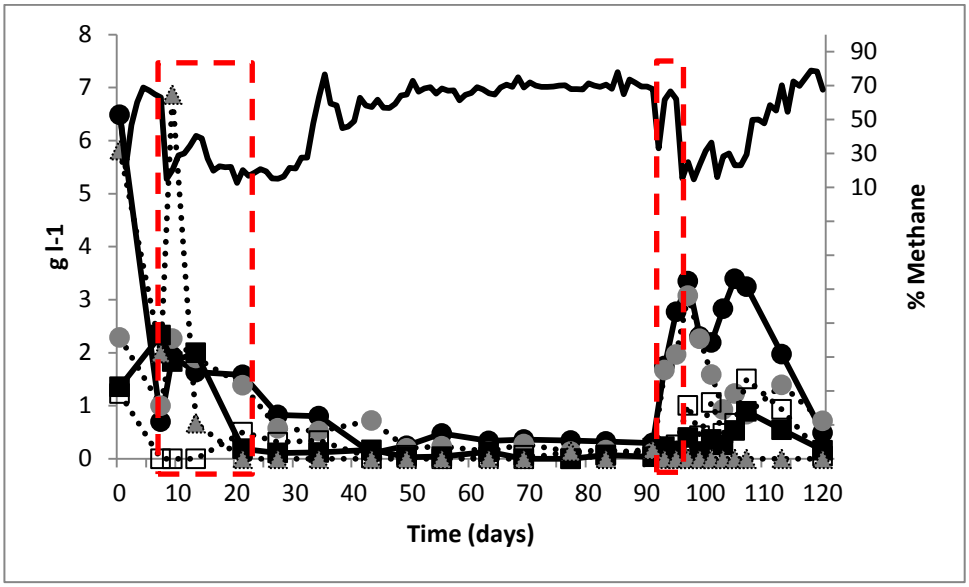

Figure 4.4. Concentrations of VFA in digesters 4-6 (exposed to both OLR increase series). Red boxes indicates OLR increase series. acetic acid (black circles and solid lines) propionic acid (black circles and dashed lines) iso butyric acid (black squares, solid lines) n butyric acid (clear squares, dashed lines) and lactic acid (grey triangles, dashed lines). Triplicate digester average, error bars are not shown to improve legibility. average standard deviation for acetic acid = 0.3, propionic acid = 0.2, iso butyric = 0.08, n butyric = 0.2, lactic = 0.1, and % methane = 4.8.

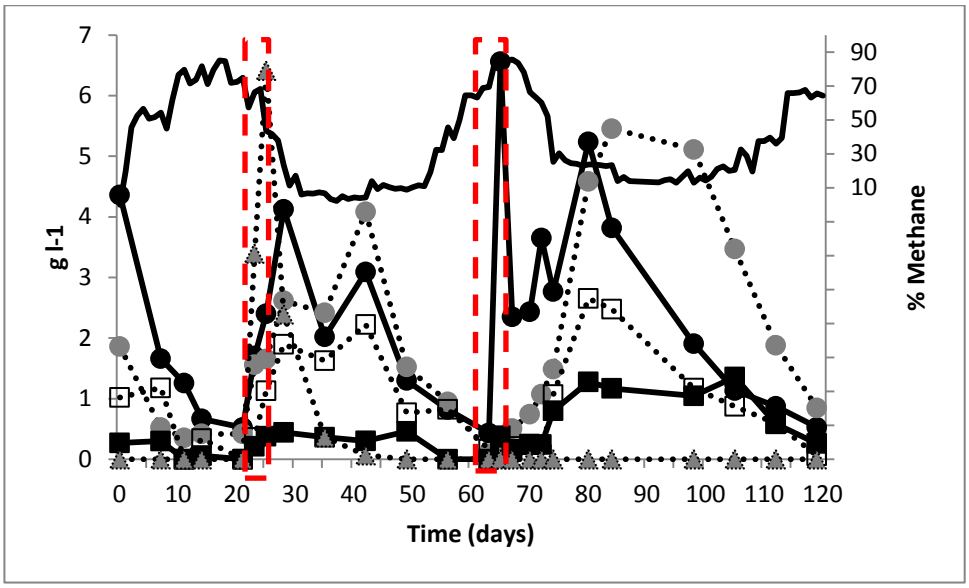

Figure 4.5. Concentrations of VFA in digesters 13-15 (Exposed to both OLR increase series). Red boxes indicates OLR increase series. acetic acid (black circles and solid lines) propionic acid (black circles and dashed lines) iso butyric acid (black squares, solid lines) n butyric acid (clear squares, dashed lines) and lactic acid (grey triangles, dashed lines). Triplicate digester average, error bars are not shown to improve legibility. average standard deviation for acetic acid = 0.4, propionic acid = 0.3, iso butyric = 0.2, n butyric = 0.1, lactic = 0, and % methane = 6.2.

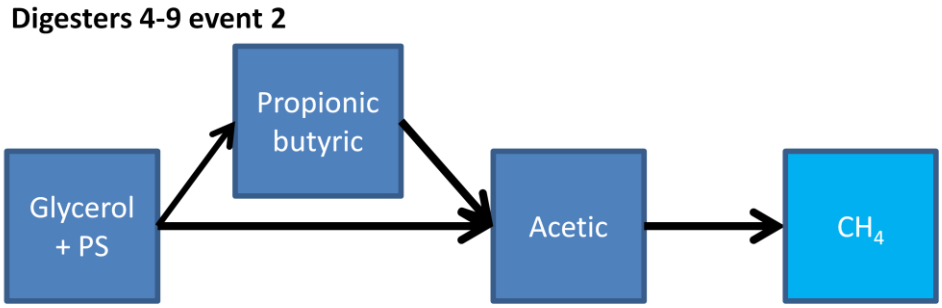

Figure 4.6. Schematic of digestion of glycerol waste during increase series 2 by digesters exposed to repeated OLR increase series with glycerol waste.

Repeated and varied OLR increase using glycerol waste: The effect of an OLR increase series using higher concentrations of glycerol waste was also investigated (digesters 7 to 9, increase series 1). The main difference between these digesters and those that received repeated OLR increase using glycerol waste at one concentration was that the production of acetic acid reached a higher concentration (5.8 ± 0.5, day 103) during the sequential phase of increase series 2, Figure 4.7. As reported in chapter 2, recovery times for biogas production and methane content were 3 days shorter in these reactors than in the one exposed to lower OLR. Although the difference was shown not to be significant ($p > 0.05$), it suggests than an even higher OLR increase in increase series 1 could result in further improved performance. A similar approach was used in previous research where much higher OLR were used to achieve higher resilience in AD microbial communities (18.8 kg VS m^3 day^{-1}) (McMahon *et al.* 2004). It is clear from these results that the effect of different OLRs on the microbial communities performances and composition needs further investigation

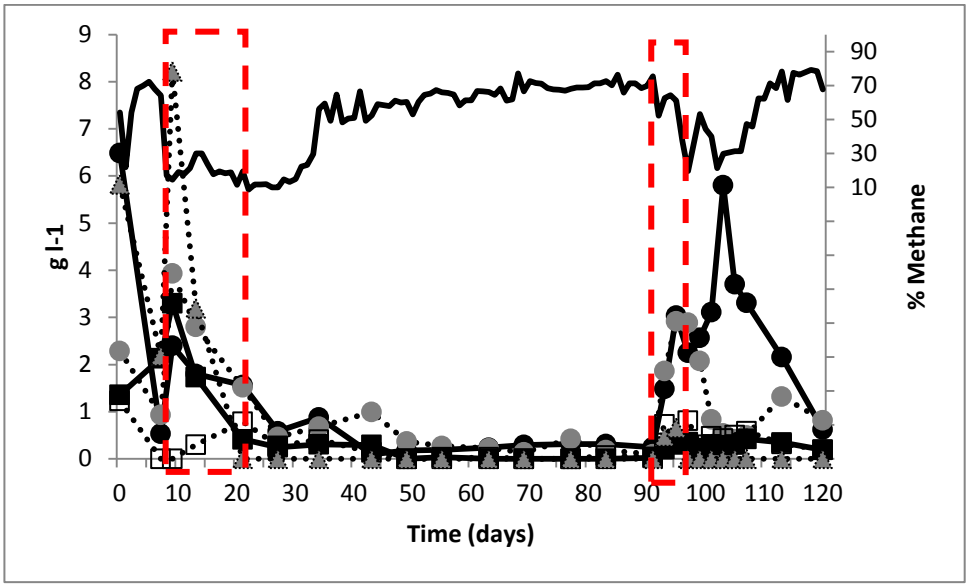

Figure 4.7. Concentrations of VFA in digesters 7-9, red boxes indicate OLR increase events, acetic acid (black circles and solid lines) propionic acid (black circles and dashed lines) iso butyric acid (black squares, solid lines) n butyric acid (clear squares, dashed lines) and lactic acid (gray triangles, dashed lines). Triplicate digester average, error bars are not shown to improve legibility. average standard deviation for acetic acid = 0.4, propionic acid = 0.2, iso butyric = 0.1, n butyric = 0.1, lactic = 0.1, and % methane = 5.6.

4.3.3. Comparison of VFA profiles.

The VFA profiles in digesters under varying conditions were compared using UPGMA cluster analysis and the Bray Curtis similarity index (Figure 4.8). There were 4 distinct groupings which further supported the analysis presented in the previous sections. Group a digesters had the highest acetic acid concentrations and comprised digesters 10-15 increase series two immediately after addition of FOGs waste (day 65). Group b had high concentrations of propionic and n butyric acid and comprised digesters exposed to glycerol waste for a second time digesters 4-9, increase series 2 and digesters exposed to FOGs waste day 98. Group c comprised digesters exposed to glycerol waste for the first time (digesters 4-9 and 13-15, increase series 1 and digesters 1-3, increase series 2) and had high concentration of lactic acid. Group d contained control digesters at the baseline OLR of 1.4 kg VS m^3 day^{-1} with low concentrations of

VFA. These results reflect the previously discussed differences in VFA production (section 3.3.1 and 2) however they can also be used to link changes in the lipid fingerprint and bacterial OTUs to the VFA profiles identified (section 3.3.4).

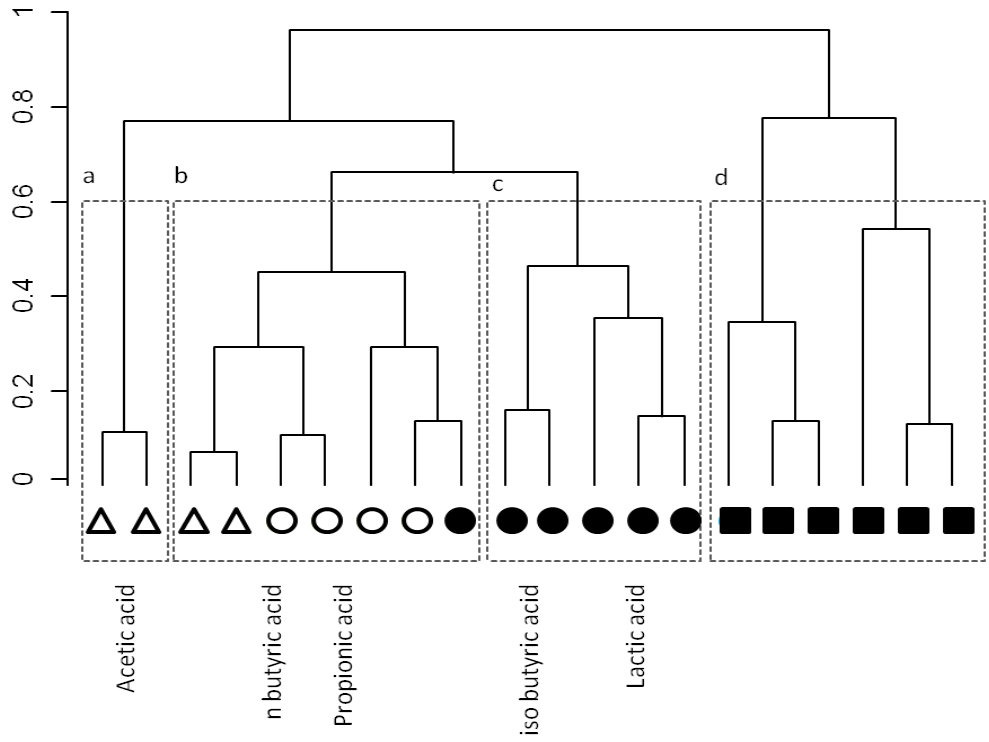

Figure 4.8. Similarity analysis of the VFA profiles of the digesters. Black circles indicate digesters during exposure to glycerol waste OLR increase series for first time, (increase series 1, digesters 4-9 and 13-15, and increase series 2 digesters 1-3) unfilled circles, exposure to glycerol waste OLR increase series for second time (increase series 2, digesters 4-9) unfilled triangles, exposure to FOGs waste OLR change (increase series 2, digesters 10-12) and black squares denote digesters under normal OLR. Profile similarities are based on UPGMA cluster analysis and the Bray Curtis similarity index, Triplicate digester averages were used for the cluster analysis. Black text denotes predominant VFAs for digester groups.

4.3.4. Links between microbial community and VFA

The relationships between the microbial community, the digesters performance, the feedstock and the OLR changes discussed in Chapter 3 showed that lower PLFA and PLEL biomass correlated with lower methane production and biogas quality. 454-

pyrosequencing analysis also revealed that periods of increased OLR resulted in a doubling of the sequences assigned to *Firmicutes*. During the single OLR increase series there was a high proportion of candidate phylum OP10 (26 %) in digesters processing FOGs waste. In contrast the digesters co-digesting glycerol waste had higher proportions of *Clostridia Incertae Sedis XV* (38 %) and *Ruminococcaceae* (17 %). Higher proportion of *Clostridia Incertae Sedis XV* in the digesters exposed to repeated increases in OLR was related to improved digester performance. It is speculated that higher the numbers of *Clostridia Incertae Sedis* XV (related to *Synergistaceae Cloacibacillus*) in the pre-exposed digesters decreased digester recovery times as it resulted in increased syntrophic interactions with the Archaea community.

Links between the VFA fingerprint, the microbial community vectors for the lipid markers and the OTUs were calculated for NMDS analysis based on the cluster analysis of the VFA profiles. The bacterial OTUs *Rumminococcaceae, Entrococcaceae, Porphymonadaceae, Prevotellaceae,* and the bacterial lipid markers iso17:1, iso15:0 and iso16:0 were related to digesters with higher concentrations of longer chain VFA such as and lactic, propionic, and iso butyric acid. The iso15:0 and iso16:0 PLFA are markers for G+ bacillus such as *Entrococcaceae* (Table 2.1) and indicate that an increase in these PLFA and *Entrococcaceae* OTUs are linked to fermentation pathways resulting in production of lactic, propionic and iso butyric acid. Therefore these bacterial OTUs/lipid markers are possible predictors for accumulation of these VFA and represent a switch in metabolic pathways other than methane production. For example, the iso-17:1 PLFA is a marker for SRB that is particularly prevalent when lactate is being metabolised during sulphate reduction (Londry *et al.* 2004). This is consistent with the results reported in chapter 3 for digesters 1-3 after the single addition of

glycerol waste (increase series 2) where an increase in the PLFA marker i17:1 and high *Enterococcaceae* representation was linked to sulphate reduction.

High concentration of acetic acid was linked to *Clostridia incerta sedis* XV (closely matched to *Synergistaceae Cloacibacillus*) the PLEL i20:0 (a general methanogenic marker) and partially to PLFA 18:1w9trans (*bacillus/clostridium* marker). In chapter 3 it was shown that the PLFA 18:1w9trans was more stable in digesters exposed to repeated OLR increase series with glycerol waste and it is theoretically related to sequential production of acetic and propionic acid and high acetic acid concentration.

Clostridia Incertae Sedis XV was shown to be closely related to *Synergistaceae* in Chapter 3. All cultivable taxa so far isolated can contribute to acidogenesis and acetogenesis via syntrophic relationships with methanogens and have been linked with lactate degradation (Baena *et al.* 1998; Delbès *et al.* 2000; Delbès *et al.* 2001; Menes and Muxí 2002; Diaz *et al.* 2007; Vartoukian *et al.* 2007). This provides further evidence that *Clostridia Incertae Sedis* XV contributed to syntrophic relationships with methanogens and resulted in improved conversion of lactic, propionic and butyric acids into acetic acid. Unknown bacterial OTUs were predominant stable digesters. This is consistent with results in chapter 3 which suggested unknown bacterial OTUs are more predominant in digesters with high biogas methane content, this will be further investigated in Chapter 5.

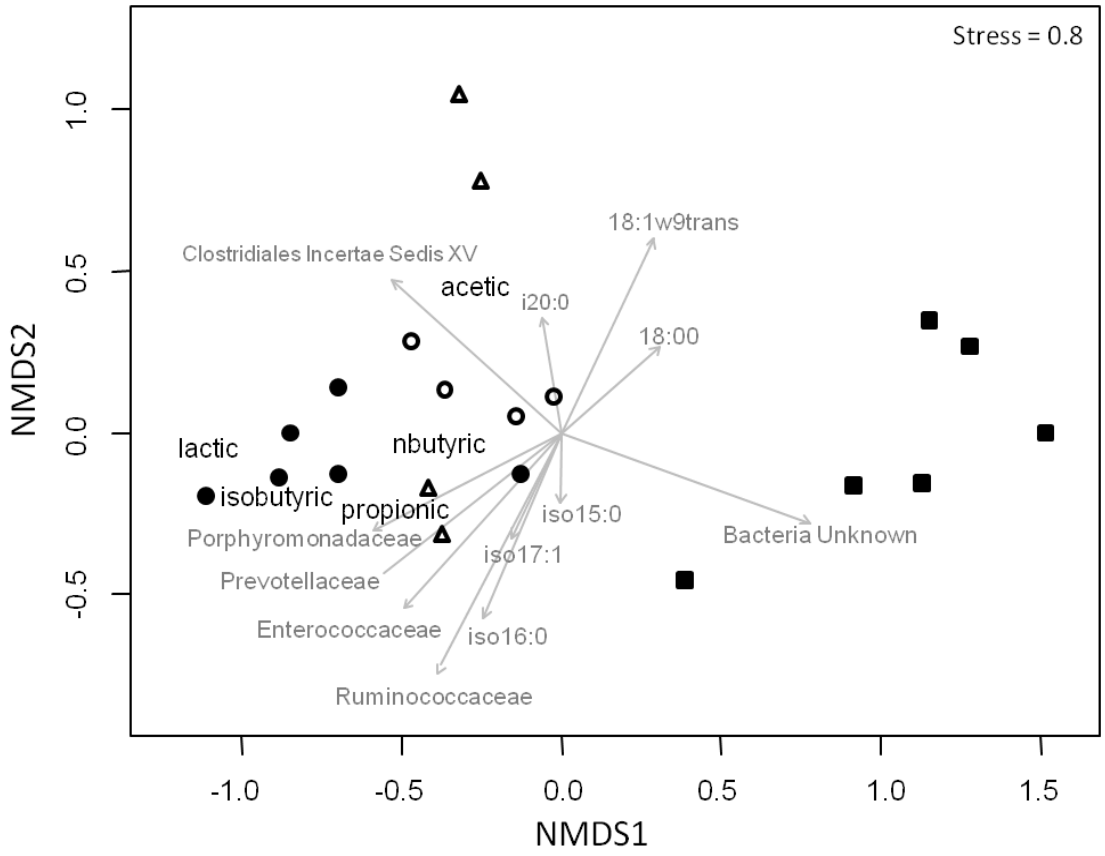

Figure 4.9. VFA profiles in digesters during exposure to glycerol waste OLR increase series for first time, black circles (increase series 1, digesters 4-9 and 13-15, and increase series 2 digesters 1-3) exposure to glycerol waste OLR increase series for second time, unfilled circles (increase series 2, digesters 4-9) and exposure to FOGs waste OLR change, triangles (increase series 2, digesters 10-12). Black squares denote digesters under normal OLR. Profile similarities are based on UPGMA cluster analysis and the Bray Curtis similarity index, 2D stress was 0.8. Triplicate digester averages were used for the cluster analysis). Black text denotes predominant VFAs for digester groups. Grey arrows show vectors for microbial lipids and bacterial OTUs based on mol % or proportion of sequences, the arrow points to the direction of most rapid change in the proportion of the lipid/OTU and the length of the arrow is proportional to the correlation between the NMDS and the lipid/OTU.

4.3.5. Implications for operational monitoring and optimisation of AD

Bioaugmentation improves recovery times during AD: In the introduction chapter it is described that if the acetic acid produced by the bacterial community cannot be rapidly processed by the archaeal community it will accumulate in the vessel, resulting

in lower pH and processing of the acid through other pathways such as hydrogen production or sulphate reduction (Van Den Berg *et al.* 1980; Conrad 1999; Schnürer *et al.* 1999; Zhang *et al.* 2007; Qu *et al.* 2009; Wang *et al.* 2009b; Laukenmann *et al.* 2011). Previous work by McMahon *et al.* (2004) reported improved resistance to OLR increase in digesters with higher numbers of syntrophic bacteria and their methanogenic partners. The results of this chapter suggest that higher numbers of *Clostridia incertae sedis* XV/*Synergistets* improved acetic acid utilisation rates and prevented processing of VFA though other pathways such as sulphate. As proposed previously these groups should be assessed as a bioaugmentation strategy for digesters which are unstable due to accumulation of VFA.

Process monitoring in AD: As discussed in chapter 2, although the links between Archaea and the concentration of VFA are well established, the relationship between bacteria and VFA composition is still unclear. As bacteria are primarily involved in the production of VFA during the acidogenic and acetogenic phases of the AD it is likely that the bacterial community structure plays a key role in regulating and controlling VFA production and their resultant conversion into methane. The bacterial families *Rumminococcaceae, Entrococcaceae, Porphymonadaceae, Prevotellaceae,* and the bacterial lipid markers iso17:1, iso15:0 and iso16:0 where predominant in digesters with high concentrations of propionic, lactic, and butyric acids. Indicating that increases in these groups could predict increase in production of longer chain VFA that are detrimental to methane production. Conversely the bacterial family *Clostridia incertae sedis* XV was linked to acetic acid production and syntrophic methanogenesis. These results are consistent with result presented in chapter 3 and provide a mechanistic

context (in terms of VFA production) to why bacterial OTUs such as *Clostridia incertae sedis* XV and *Rumminococcaceae* can be used to predict AD performance.

4.4. Conclusions

In chapter 2 and 3 it was shown that a shift in the bacterial community structure was related to the faster recovery in digesters exposed to repeated OLR changes with glycerol waste. In this chapter it is possible to link this shift to a change in the way organic acids are processed. When glycerol waste was used as co-digestant, digesters with not previously exposed to glycerol waste (single OLR increase series) produced lactic acid as the main organic acid, followed by parallel production of acetic and propionic acid. There was also evidence that sulphate reduction was a major pathway in these digesters, resulting in hydrogen sulphide production over methane. The digesters exposed to repeated OLR change with glycerol waste showed minimal lactic acid production and switched to sequential production of acetic and propionic acid, resulting in high concentrations of acetic acid and production of methane.

When FOGs waste was used as co-digestion feedstock, the production of VFA was the same for digesters that were exposed to repeated or single OLR increase series. The digestion could be divided into two main stages. Stage one was characterised by accumulation of acetic acid and high biogas production and stage two was characterised accumulation of propionic acid and poor biogas production. Microbial communities were shown to be different in the two digester groups. This highlights that both the function of and structure of the microbial community are important when trying to achieve AD optimisation as changes in microbial community structure are not always related to changes in performance.

The results also established links between the bacterial community structure and VFA profiles, the bacterial families *Rumminococcaceae, Entrococcaceae, Porphymonadaceae, Prevotellaceae,* and the bacterial lipid markers iso17:1, iso15:0 and iso16:0 were linked to the production of lactic, propionic and iso butyric acid. Conversely the bacterial family *Clostridia incertae sedis* XV and the archaeal lipid marker i20:0 was linked to acetic acid production. This confirms that, as hypothesised in chapter 3, syntrophic relationships between *Clostridia incertae sedis* XV and Archaea can result in a reduction of the concentrations of other VFA and a more efficient methanogenesis and provides a mechanistic context to the faster recovery observed in chapter 2.

Chapter 5: Microbial community dynamics and anaerobic digester performance

Abstract: In this chapter the structure and composition of microbial communities of anaerobic digesters exposed to different co-digestion substrates and varying biogas methane content and organic loading rate (OLR) were investigated using 454-pyrosequencing and lipid fingerprinting analysis. Lipid fingerprinting demonstrated that microbial biomass decrease was correlated with reduced methane content. 454-pyrosequencing analysis further showed that decrease in biogas methane content to less than 30 % was correlated with a 50% increase of *Firmicutes* sequences (particularly in OTUs related to *Ruminococcus*). The bacterial community in digesters with the highest biogas methane content (71 + %) had a high number of sequences related to unidentified bacterial OTUs (> 45 %) highlighting how important further research is needed to better understand the diversity dynamics of AD. pH and methane content were also significantly positively correlated with community evenness suggesting that even distribution of the microbial groups was related with AD performance. The findings further highlighted a number of OTUs including *Syntrophomonas* and *clostridium incertae sedis* XV */Aminobacterium* that should be investigated for bioaugmentation of AD.

5.1. Introduction

Although anaerobic digestion (AD) is already a widely implemented technology for the treatment of wastewater and organic mixed solid wastes, poor anaerobic digester performance and system failure are still common issues. Most of these problems occur as a result of inadequate operational and process control and a lack of understanding of the dynamics of the microbial processes taking place in the digesters (Leitao *et al.* 2006). This has contributed to a lack of investors and low regulatory confidence in the technology, creating a barrier to establishing a market. There is now a general consensus among the scientific community that in-depth understanding of the microbial ecology of AD is vital to optimise and manage adequately the process (Briones and Raskin 2003; Rittmann 2006; Rittmann *et al.* 2006; Werner *et al.* 2012). Despite this, plant operators often treat microbial ecology as a 'black box' and optimisation and management of AD is mainly based on the physicochemical parameters of AD. Developments in culture independent molecular methods have led to a number of studies analysing the microbial communities in AD, both at laboratory scale (Delbès *et al.* 2000; Kaewpipat and GradyJr 2002; Sundh *et al.* 2003) and full-scale reactors (LaPara *et al.* 2000; Angenent *et al.* 2002; McMahon *et al.* 2004; Karakashev *et al.* 2005; Keyser *et al.* 2006). Most of these studies demonstrated that the microbial ecology of AD is highly diverse and dynamic. Unstable communities have been observed in digesters with stable performance, rendering it difficult to formulate any generic trends/relationships between microbial community response and digester performance (Fernández *et al.* 1999; Zumstein *et al.* 2000; Wang *et al.* 2010b; Wang *et al.* 2011). Recently studies of the microbial ecology of wastewater anaerobic digesters showed that it was possible to link digester performance with fundamental ecological

relationships such as community evenness, which is a measure of how close in numbers each species in a community are, and ecological theories such as the biogeography model, the species-area relationships and the taxa-time relationships (Wells *et al.* 2011; Valentin-Vargas *et al.* 2012; Werner *et al.* 2012). Therefore, the possibility of integrating the engineering of anaerobic digesters with microbial ecological theories are now a genuine prospect (Briones and Raskin 2003; Rittmann 2006).

It is clear that to understand and optimise AD, a detailed examination of the microbial ecology needs to be carried out to consolidate the information collected to date. Towards this there are a number of key questions that need to be addressed including i) are there any consistent changes in community structure correlated with AD performance? ii) are there particular groups of bacteria that are associated with the optimal performance of digesters?, and iii) are there any relationship between performance and fundamental ecological parameters such as community evenness ?

Molecular based lipid fingerprinting and PCR-based 454-pyrosequencing analyses were carried out to investigate the microbial communities structure, biomass and dynamics in the digesters. Molecular based lipid fingerprinting analysis provided insights into the microbial biomass changes and microbial community structure, in the digesters. However a major limitation of the lipids analysis is the low taxonomic resolution and the poor resolution of rare diversity as unusual lipid are not quantified (Frostegård *et al.* 2011). To overcome this 454-pyrosequencing was used to gain detailed phylogenetic information on both the dominant and minor important members of the microbial community. A number of studies have shown the value of this technique in understanding AD (Schlüter *et al.* 2008; Kröber *et al.* 2009; Lee *et al.* 2012; Werner *et al.* 2012). Samples were collected from anaerobic digesters at two scales, with varying

co-digestion substrates and operational conditions. This was done to understand whether changes in the microbial communities where specific to one set of conditions or applicable at a wider scale. To further determine if specific groups were associated with digester performance, the digesters were grouped based on methane production ranging from 0-30, 31-60, and 61-85 %. Unique Operational Taxonomic Units (OTUs) to each group were determined to identify a core group of bacteria associated with high methane production. Regression analysis was carried out to determine the relationships between microbial community evenness and anaerobic digesters performance.

5.2. Methods

5.2.1. Digester operational parameters

Laboratory-scale semi-continuous digesters consisted of 1-L borosilicate glass bottles with a 700 ml working volume for the co-digestion of glycerol waste; and 5-L bottles with a 4.5 L working for all other conditions (Table 2.1). A temperature of 38°C was maintained inside the digesters by emersion in a Perspex water bath with temperature regulated by two Grant G120 water heaters (Grant Instruments Ltd. Cambridge. UK). Digesters were seeded with secondary sludge from the AD plant of a local Sewage Treatment Works (Milton Keynes, UK). Digesters were fed with autoclaved primary sludge were fed three times a week to achieve a retention time of 7 days. Changes in feed and OLR are outlined in chapter 2 and table (Table 2.1). For the analysis in this chapter the digesters were split into two sets as follows: set 1) digesters 1-9, one L digesters with a working volume of 700 ml: and set 2) digesters 10-15, 5-L digesters with a working volume of 4.5 L.

5.2.2. Biogas production, methane concentration and physicochemical characterisation

Gas production was measured daily by water displacement in a glass column (150 x 5 cm, green food dye was used to aid measurement). Volumes are corrected to standard atmospheric conditions (25 °C and 1 bar). Methane content was measured using a SERVOPRO1400 CH_4 gas analyser (Servomex, UK) according to manufacturer recommendations. pH and alkalinity were measured according to standard APHA methods (APHA 1989). Propionate : Acetate (P:A) ratio was calculated as the ratio between propionic and acetic acid concentration measured by HPLC analysis,. A ratio of greater than 1.4 is considered an indicator of imminent digester failure (Hill *et al.* 1987).

5.2.3. Volatile fatty acids analysis

Detailed methods for the Phospholipids VFA analysis are given in Chapter 2.

5.2.4. Phospholipids and ether-linked isoprenoids analysis (PLFA and PLEL).

Detailed methods for the PLFA and PLEL analysis are given in Chapter 3.

5.2.5. Taxonomic affiliation of PLFA and PLEL

The taxonomic affiliations are summarised in Table 5.1 as described in Chapter 3.

Table 5.1. Summary of PLFA and PLEL taxonomic affiliation (adapted from Gattinger *et al.* 2002; Gattinger *et al.* 2003; Londry *et al.* 2004; Oravecz *et al.* 2004; Radl *et al.* 2007)

		Taxonomic affiliation
Straight saturated		
11:0	ubiquitous	
12:0		
13:0		*δ-Proteobacteria*
14:0		*Actinobacteria/ δ-Proteobacteria*/ low GC G+
15:0		*Actinobacteria*
16:0		*Actinobacteria*/ low GC G+/CFB/ *δ-Proteobacteria*
17:0		Δ-*Proteobacteria*
18:0		low GC G+ *Bacillus/ Clostridium*
20:0		
iso and anteiso branched		
iso-15:0	G+	low GC G+/*Bacillus/ Actinobacteria*
anteiso-15:0		*Actinobacteria*/ *Bacillus*/ low GC G+/CFB[a]/ δ-*Proteobacteria*
iso-16:0		low GC G+/ *Bacillus/ Actinobacteria*
iso-17:1		CFB (*Cytophaga*)/SRB[b]
cyclopropane		
cyc 17:0	G-	Anaerobes/ *Bacillus/ Clostridium* / δ-P*roteobacteria*
cyc-19:0		Anaerobes/ *Bacillus/ Clostridium* / δ-*Proteobacteria*
mono-unsaturated		
16:1 w7cis	G-	*Bacillus/ Clostridium*
18:2 w6cis		*Actinobacteria* / *Bacillus/ Clostridium*
18:1 w9cis		Anaerobes/ *Bacillus/ Clostridium*
18:1w9trans		Anaerobes/ *Bacillus/ Clostridium*
poly-unsaturated		
18:2w6,9		Fungi
hydroxy		
2OH-10:0	G-	*δ-Proteobacteria*
2OH-12:0		*δ-Proteobacteria*
3OH-12:0		*δ-Proteobacteria*
3OH-14:0		*δ-Proteobacteria*
2-OH 16:0		*δ-Proteobacteria*/CFB
isoprenoids		
i20:0	Methanogens	*Euryarchaeota*
i20:1		*Methanosarcina*
i40:0		Hydrogenotrophic methanogens

[a] CFB: *Cytophaga-Flavobacteria-Bacteroides* [b]SRB: sulphate-reducing bacteria

5.2.6. 454-pyrosequencing analysis and Bioinformatics

Detailed methods for 454-pyrosequencing analysis and Bioinformatics are given in Chapter 3.

5.2.7. Statistical analysis.

Pielou's community evenness index (*J'*) was calculated according to the formula $J' = H'/H'max$, where *H'* is the Shannon diversity index and *H'* max is the maximum value of *H'*. *J'* is constrained between 0 and 1 with 1 = a completely even community. Linear regression between evenness and methane biogas content was then carried out using the R project for statisitivl computing. One-way analysis of similarity (ANOSIM) was carried out with Primer software beta version 6 (PRIMER-E Ltd, UK) according to Clarke and Gorley (2006)

5.3. Results and discussion

5.3.1. Digester operational conditions

In order to identify whether there are any trends, correlation between microbial community structure and dynamics and digesters performance independently of the type of feedstocks used, the digesters were grouped according to the methane content of their biogas (Table 5.2). Overall 6 groups were identified and further correlated to VFA production, alkalinity and V/A ratio.

Table 5.2. Summary of the key physico-chemical parameters of the digesters.

Digester group	No. of samples	%CH_4	pH	Alkalinity ($gCaCO_3\ l^{-1}$)	Volatile fatty acids ($g\ l^{-1}$)						V/A ratio*
					Total VFA	Acetic acid	Propionic acid	Butyric acid		Lactic acid	
								iso	n		
1	7	0 – 20	5.8	1	8 ± 2	2 ± 1	4± 1	1.1 ± 2	0.8 ± 2	1.3 ± 2	8 ± 3
2	7	21- 30	5.9	1.1	6.3 ± 1	2.6 ± 1	1.9 ± 1	0.9 ± 1	0.7 ± 0.6	0.7 ± 1	5.6 ± 1
3	3	31- 45	6.5	1.3	6.4 ± 3	2.7 ± 1	1.8 ± 1	1.0 ± 1	0.7 ± 0.4	1.6 ± 1	5.4 ± 0
4	5	46 – 60	6.9	2.2	2.6 ± 2	0.9 ± 1	0.7 ± 1	0.3 ± 1	0.2 ± 0.3	0.1 ± 1	0.9 ± 0
5	9	61- 70	7.2	2.4	1.8 ± 1	0.6 ± 1	0.7 ± 1	0.2 ± 1	0.3 ± 0.3	0.5 ± 0	1.1 ± 0
6	5	>70	7.3	3.0	2.2 ± 2	0.3 ± 0	0.7 ± 0.9	0.0 ± 0	0.04 ± 0	0.0 ± 0	0.4 ± 0

*Ratio of VFA/total alkalinity

5.3.2. Relationships between AD performance and molecular based lipid fingerprinting

Many of the lipid markers were associated to *Actinobacteria*, low GC Gram positive bacteria, CFB, δ-*Proteobacteria*, *Bacillus*, and *Clostridia* (Table 3.1). There were relatively little changes in the contribution of many of the individual PLFAs to the total fingerprint at different biogas methane content. The only PLFAs that varied significantly were the PLFA18:1w9cis and 18:1w9trans (Table 5.3). The trans oleic acid (18:1w9trans) doubled in concentration when methane content was ≥ 60% in comparison to digesters with a methane content ≤ 20 %. In contrast, the cis oleic acid (18:w9cis) doubled in digesters with a methane content ≤ 30 %. This finding suggests changes in the community structure and/or metabolic function of the associated bacterial groups (Anaerobes/ CFB group). No change in the ratio of cyclopropyl to mono-unsaturated fatty acids (cy17:0/16:1ω7c and cy19:0/18:1ω7c) was observed suggesting that the microbial community of the digesters were not experiencing significant stress conditions despite varying the OLR of the digesters (Frostegård *et al.* 2011). The PLFA 18:2w6,9 which is associated with fungi made up approximately 3.5 for Mol % in all digesters conditions. Fungi are not often considered in studies of the microbial communities in AD (not targeted by primers) and their potential role in AD remains to date unrevealed. It is possible that they could play a role in cellulose digestion as this has been observed in cattle rumen, therefore fungi could be a key hydrolytic group in AD (Bauchop 1981; Bauchop and Mountfort 1981). It has been shown that fungi can survive in AD process and due to possible health risks, particularly if the digestate is used as fertiliser or stored in the open air, they must be considered in future research (Schnürer and Schnürer 2006; Pankhurst *et al.* 2012).

Table 5.3. Summary of PLFA and PLEL (mol %) in digesters with varying biogas methane content. Taxonomic affiliations given in Table 5.1. superscript numbers are standard deviation, number of samples is as indicated in Table 5.2.

Lipid	**Digester group (% methane)**									
	0 - 20		21 - 30		31 - 45		46 - 60		61 - 70		>70	
11:0	1.7	0.3	1.5	1.4	0.6	0.4	0.8	0.2	0.6	0.4	0.4	0.3
12:0	0	0.0	0	0.2	0	0.3	0	0.0	0	0.1	0.1	0.0
13:0	0.1	0.0	0.2	0.0	0.1	0.0	01	0.0	0.1	0.0	0.1	0.1
14:0	3.6	0.0	3.6	0.2	2.9	0.1	4.2	0.0	2.8	0.1	2.7	0.1
15:0	1.6	0.0	1.6	0.0	1.5	0.0	1.6	0.0	1.4	0.0	1.5	0.0
16:0	16.8	0.0	16.3	0.0	16.5	0.0	16.7	0.0	15.8	0.0	16.8	0.4
17:0	0.6	0.4	0.5	2.3	0.4	0.9	0.6	0.8	0.5	0.7	0.4	0.9
18:0	7.6	0.2	7.7	4.0	9.9	3.1	8.8	1.2	10.3	2.5	10.7	3.2
20:0	0.4	0.3	0.4	2.9	0.4	3.5	0.3	1.7	0.4	2.6	0.3	2.9
iso-15:0	9.3	0.1	8.7	0.9	8.9	0.5	9.5	0.2	8.5	0.4	8.4	0.5
a-15:0	8.5	0.0	7.9	0.7	9.1	0.9	9.8	0.4	8.7	0.6	8.5	0.6
iso-16:0	1.8	0.0	2.2	2.4	0.7	0.2	1.2	0.4	1.0	0.5	1.4	1.1
iso-17:1	1.0	0.4	0.9	0.7	0.9	5.8	1.0	2.9	1.2	4.5	0.9	4.0
cyc-17:0	0.2	0.0	0.2	4.3	0.2	3.1	0.0	3.1	0.4	5.1	0.4	3.3
cyc-19:0	0.4	0.0	0.4	0.5	0.3	0.5	0.4	0.2	0.3	1.0	0.3	0.3
16:1 w7cis	14.2	0.0	12.0	0.0	14.6	0.0	16.3	0.0	14.5	0.3	14.1	0.3
18:2 w6cis	15.3	0.0	14.7	0.3	12.6	0.1	12.5	0.1	12.9	0.2	13.1	0.2
18:1 w9cis	5.4	0.0	5.5	0.4	3.0	0.6	4.1	0.0	2.4	0.5	2.2	0.6
18:1w9$_{trans}$	6.9	4.3	9.8	0.6	12.3	1.7	10.8	0.3	12.8	1.7	12.9	1.3
18:2w6,9	4.0	0.2	3.8	3.2	3.2	2.6	0.0	1.9	4.1	2.9	3.5	4.2
2OH-10:0	0.0	1.7	0.1	3.2	0.1	3.6	0.0	3.2	0.0	3.4	0.0	3.4
2OH-12:0	0.0	0.6	0.0	1.8	0.1	2.7	0.0	1.9	0.1	2.0	0.1	2.9
3OH-12:0	0.0	0.0	0.0	0.2	0.0	0.1	0.0	0.1	0.0	0.1	0.2	0.1

Similarity of the PLFA profiles (based on mol %) of the digesters groups showed there are two distinct groups at > 90% similarity (Figure 5.1) All the digesters with > 31 % biogas methane content are > 90 % similar to each other as are those with < 30 % biogas methane content. ANOSIM showed that the differences between group 1 and group 2 were significant at p = 0.02 and R = 0.23. The low value of R reflects that the differences in PLFA fingerprint were confined to a only a few of the PLFA markers, specifically the 18:1w9trans and cis as discussed. This is consistent with previous research that showed that increasing biogas production is correlated with distinct changes in PLFA profiles and therefore lipid fingerprinting could be used to monitor AD performance (Schwarzenauer and Illmer 2012).

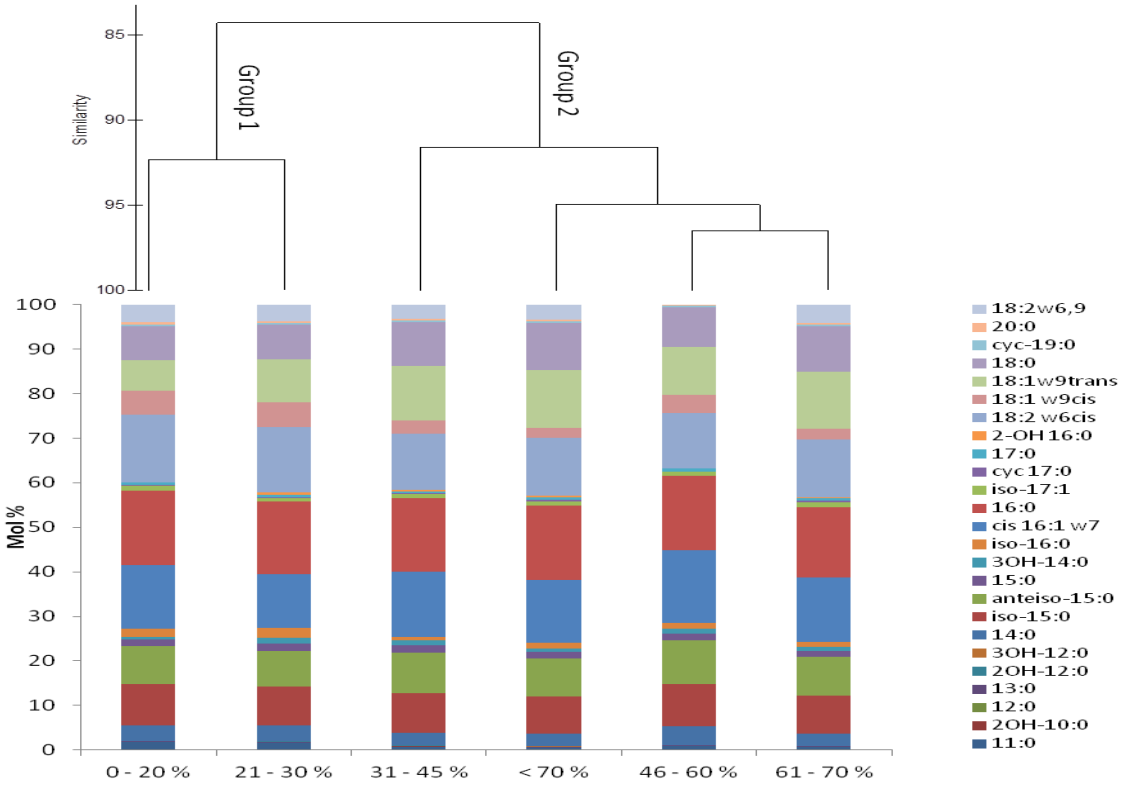

Figure 5.1. Similarity analysis of the bacterial community structure of the different digesters groups based on PLFA fingerprint. The UPGMA cluster analysis was performed using log transformation of PLFA data and the Bray Curtis similarity index. The bar chart shows the mol % of PLFA that made up > 2 % of the PLFA fingerprint.

In Chapter 3 it was demonstrated that PLFA and PLEL concentrations were significantly correlated with methane concentration. Further to this the total concentration of PLFA identified in the different groups of digesters showed that is the average concentration of PLFA was clearly different between the two trials Figure 5.2. However similar trend was observed, as methane content increases, bacterial biomass increases (three times increase in Experiment 1 and 1.5 times increase in Experiment 2). This reflects that FOGs waste addition in Experiment 2 did not affect the PLFA concentration (as discussed in Chapter 3). The methanogenic biomass increased by one order of magnitude as the % methane in biogas increases Figure 5.3 which was expected as the methanogens are responsible for all methane production and do not have other metabolic pathways available (Garcia *et al.* 2000).

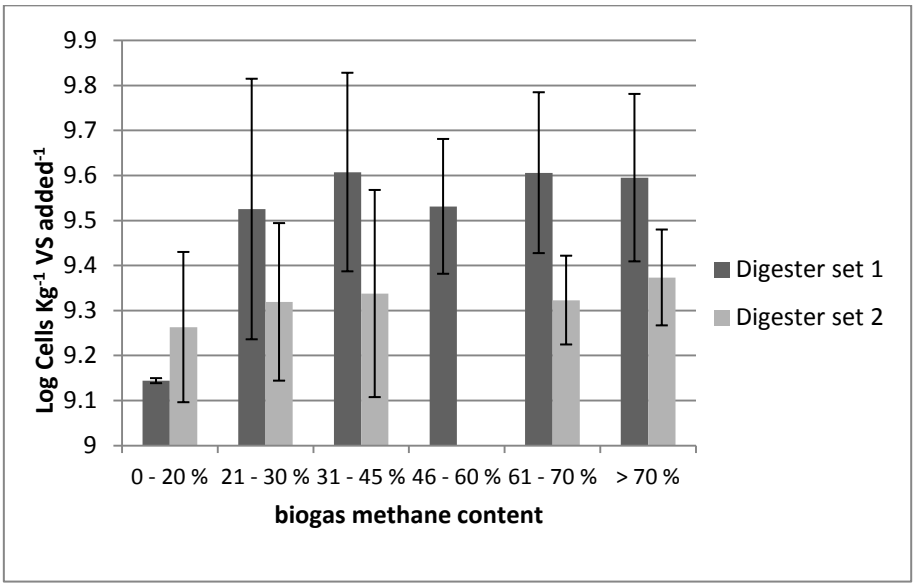

Figure 5.2. Bacterial biomass in digesters grouped by biogas methane content in both digester sets. Error bars represent standard deviation.

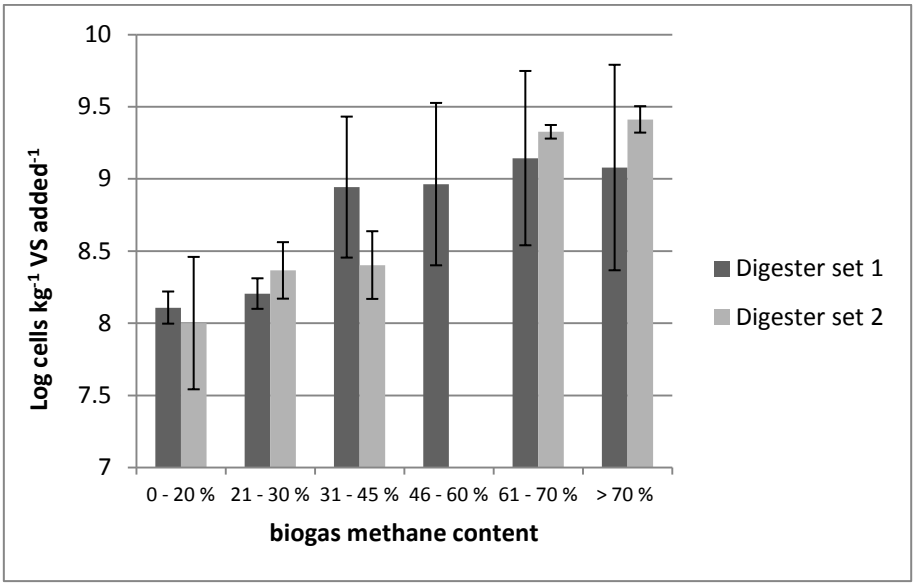

Figure 5.3. Concentrations of dominant PLEL in digester grouped by biogas methane content. Error bars represent standard deviation.

5.3.3. Microbial community diversity and dynamics

Due to the low numbers of quality sequences obtained for the Archaea, most of the analysis concentrates on bacterial community structure changes and dynamics. However, section 5.3.8 gives a summary of the archaeal data. A total of 19,363 bacterial sequences were clustered into 1334 OTUs. The rank abundance curve revealed that 17 % of the total OTUs were comprised of > 5 sequences (Figure 5.4). This indicates that there was a core group of approximately 15 % of OTUs present across the whole data set. The OTUs were assigned to *Firmicutes* (57 %) *Bacteroidetes* (15 %) *Proteobacteria* (12 %) and unknown (10 %). Predominance of these bacterial classes is consistent with results reported in other studies using NGS techniques (Schlüter *et al.* 2008; Kröber *et al.* 2009; Lee *et al.* 2012; Werner *et al.* 2012).

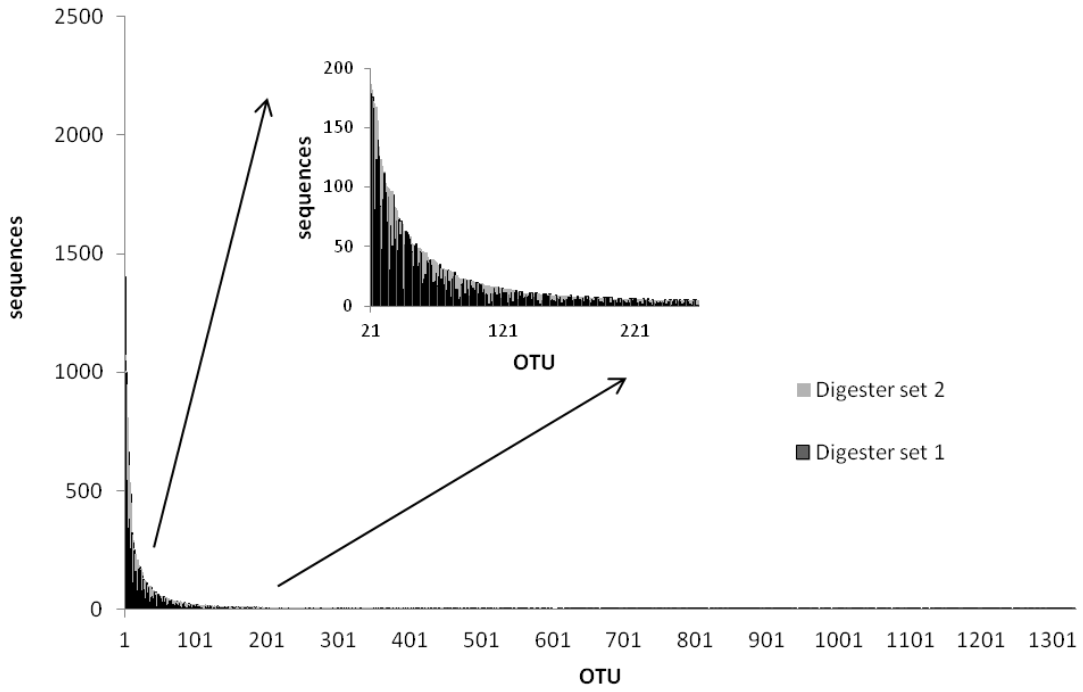

Figure 5.4. OTUs established for sequences obtained from 454- pyrosequencing

5.3.4. Relationships between AD performance and structure of the bacterial community at high taxon level

Comparison of the bacterial communities based on the numbers of OTUs for each digester group clearly demonstrates a shift in the structure of the communities as methane content increases (Figure 5.5). Digesters with a methane content ≥70 % were clearly distinct from all other groups. As observed with the lipid analysis, two groups of digesters were identified with a similarity ≥ 80 %, including digesters with 0-30 % methane content and those with 31-70 % (Figure 5.5).

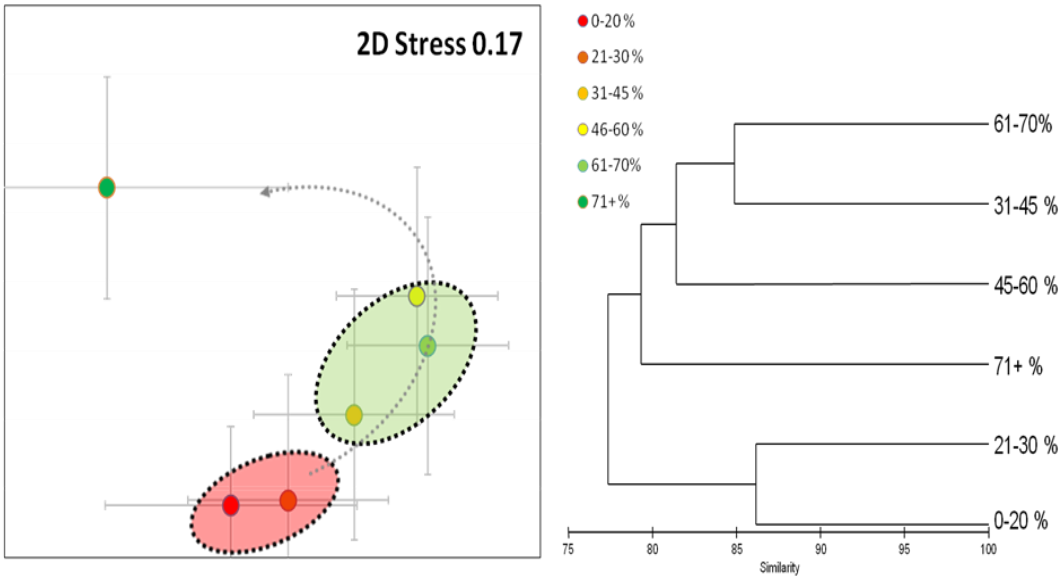

Figure 5.5. Comparison of the bacterial communities structure in the digesters based on the number of sequences assigned at order level to digester groups. UPGMA cluster analysis on the left side and 2D-nMDS configuration on the right side. Clusters are delineated by taking a similarity of 80 % and the dashed arrow shows the trajectory of increasing biogas methane content.

The relative proportion of each bacterial order contributing to more than 5% of the total microbial communities based on the number of sequences assigned to that taxonomic group is shown in Figure 5.6. These orders represented on average 94 % of the diversity in the digesters. *Clostridales* which represent more than 40 % of the sequences identified are dominant in digesters with a methane content ≤ 30 %. As methane content increases, the number of *Clostridales* decreased. Digesters with low methane concentration also had high concentration of VFA (Table 5.2). This finding suggests that the high levels of *Clostridia* are related to an increase fermentation processes in these digesters. This confirms the hypothesis of Chapter 3 that increase in *Clostridales* is correlated with poor digester performance. *Bacteroidales* barely changed making up 20 – 30 % of the community at all levels. The number of sequences of unidentified bacteria increased threefold in digesters with a biogas methane content >70%. This

could be explained by high specialism in AD methane producing bacterial communities. As a result of this many of the groups are yet to be cultured and/or remain unidentified in the databases with unknown function (Narihiro and Sekiguchi 2007). This highlights that further work is still needed to better characterise the diversity of AD and improve our understanding of the microbial dynamics in response to operational conditions changes.

The candidate phylum OP10 was highly represented (> 10 %) in digesters with 31-45 % and > 70% biogas methane content Figure 5.6. The phylum OP10 was, until recently only based on PCR-based analysis of 16S rRNA genes from environmental samples (Portillo and Gonzalez 2009). Sequences assigned to OP10 have been collected from a diverse range of environments including air particles, bioreactors, hot springs, sludge, and soil and have been classified as a new phylum *Armatimonadetes*. Three species have also now been isolated including *Fimbriimonas ginsengisoli*, *Armatimonas rose*, and *Chthonomonas calidirosea*; (Lee *et al.* 2010; Tamaki *et al.* 2010; Im *et al.* 2012; Lee *et al.* 2013). To date none of these species have been reported in AD systems so their precise role is uncertain. However they all have an optimal pH in the range of 5.3-6.5 and digest a range of mono-, di- and poly-saccharides. This suggests that they can be involved in the hydrolysis and acidogenesis. In Chapter 3 they were identified in digesters co-digesting FOGs. When considering the range of metabolic diversity in other Phyla it would be premature to assign a specific role to the OP10/*Armatimonadete*s in AD based only on three species (Lee *et al.* 2010; Tamaki *et al.* 2010). This again highlights our limited understanding of the structure of microbial communities in AD and also the importance of gaining a better understanding of the

function of these bacteria (which is often overlooked). *Proteobacteria* numbers were constant, representing approximately 5 % of the total microbial communities Figure 5.6. The *Beta-proteobacteria* dominated at all levels of biogas methane content (Figure 5.7); in particular they constituted 90 % of the total *Proteobacteria* in digesters with 45-60 % biogas methane content. In digesters with 31-45 % biogas methane content, their contribution decreased to 38 % with a concomitant increase of the *Gamma-proteobacteria* to 38 %.

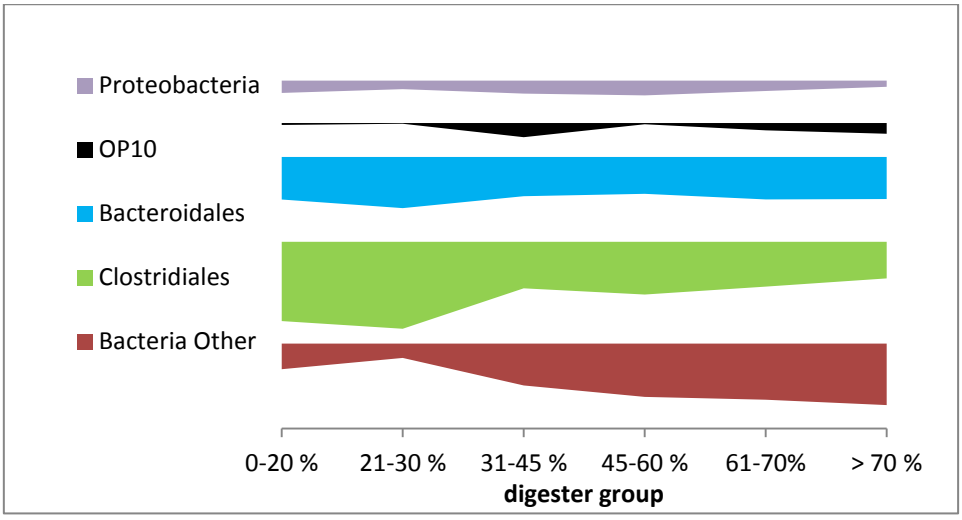

Figure 5.6. Relative proportions of the dominant orders based on number of sequences assigned to that taxonomic group. The groups are given at order level excluding Proteobacteria which are given as phylum and then summarised at order in Figure 5.7.

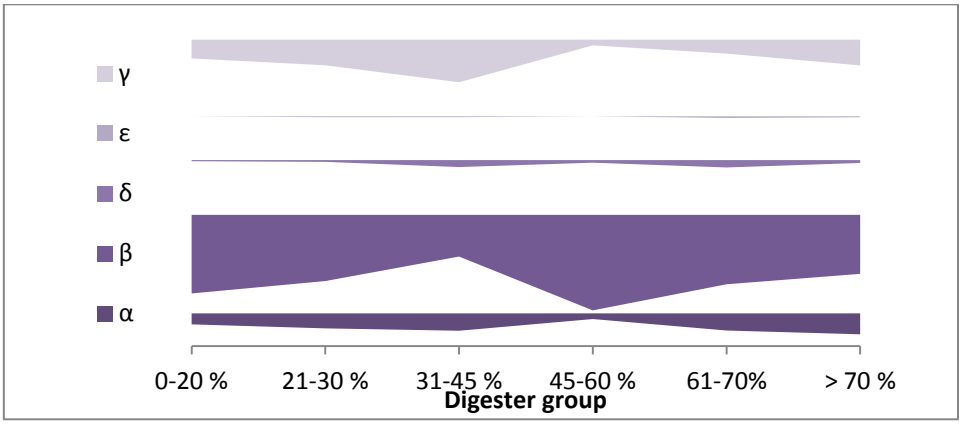

Figure 5.7. Relative distribution of *Proteobacteria* classes based on % of sequences assigned to that taxonomic group.

5.3.5. Identification of the dominant bacterial OTUs and relationships with methane content.

The results presented previously showed that the lipid fingerprints of the digesters and the changes in the proportions of the higher bacterial taxons were correlated with methane content was to. As shown in Figure 5.4 there was a core group of dominant OTUs. Therefore an analysis was carried out to see if these OTUs could be linked to digester performance. Among the 40 most dominant OTUs (Table 5.4), the relative abundance (numbers) of 16 OTUs clearly changed according to methane content of the digester groups from low to high biogas methane content (Figure 5.8).

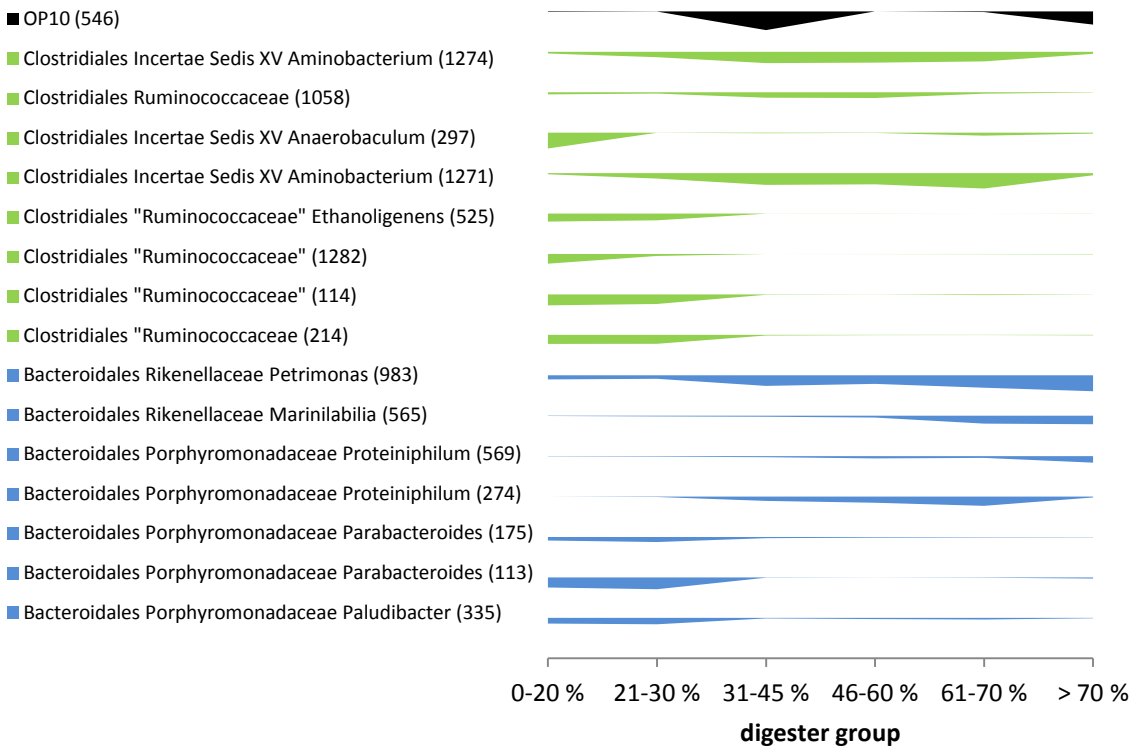

Figure 5.8. Relative number of sequences assigned to dominant OTUs that varied according to methane content of the digester groups

Table 5.4: Summary of the 40 most represented OTUs.

Cluster	Higher Taxon	Order/Family/Genus	% Match	Closest relative	Probable function
1385	*Thermotogae*	*Thermotogae/Thermotogales/Thermotogaceae/Petrotoga*	94	FR850164	hydrolysis
317	*Proteobacteria*	*Betaproteobacteria/Rhodocyclales/Rhodocyclaceae/Azospira*	92	EF515439	nitrogen fixation
546	*OP10*	*Armatimonadales/ Armatimonadaceae/ Armatimonas*	65	AJ009490	unknown
1505		*Unknown*	87	AB175378	unknown
432		*Clostridia/Clostridiales/Veillonellaceae*	83	GQ167189	acidogenesis
1058		*Clostridia/Clostridiales/Ruminococcaceae*	70	GU559795	Hydrolysis/ acidogenesis
597		*Clostridia/Clostridiales/Peptostreptococcaceae*	85	FJ660527	hydrolysis (protein)
297		*Clostridia/Clostridiales/IncertaeSedisXV/Anaerobaculum*	89	AB274507	hydrolysis (protein)
1271		*Clostridia/Clostridiales/IncertaeSedisXV/Aminobacterium*	89	AF073521	hydrolysis (protein)
1274	*Firmicutes*	*Clostridia/Clostridiales/IncertaeSedisXV*	93	AF280863	Hydrolysis/acid ogenesis
402		*Clostridia/Clostridiales/IncertaeSedisXI/Soehngenia*	83	AF395426	Hydrolysis (carbohydrate)
243		*Clostridia/Clostridiales/IncertaeSedisXI/Sedimentibacter*	96	AY221992	acidogenesis
1275		*Clostridia/Clostridiales/ Clostridiaceae1/Clostridium*	83	HQ904236	Hydrolysis /acidogenesis
733		*Clostridia/Clostridiales*	67	GU124470	Hydrolysis/ acidogenesis
1381		*Clostridia/Clostridiales*	70	EF063632	Hydrolysis/ acidogenesis
525		*Clostridia/Clostridiales/Ruminococcaceae /Ethanoligenens*	63	DQ444185	acidogenesis

1282		*Clostridia/Clostridiales/Ruminococcaceae*	94	EU741666	Hydrolysis/ acidogenesis
114		*Clostridia/Clostridiales/Ruminococcaceae*	72	AJ404681	Hydrolysis/ acidogenesis
214		*Clostridia/Clostridiales/Ruminococcaceae*	64	AY330124	Hydrolysis/ acidogenesis
1485		*Clostridia/Clostridiales/Eubacteriaceae*	90	AF427914	Hydrolysis/ acidogenesis
278	*Chloroflexi*	*Caldilineales/Caldilineacea/Levilinea*	87	Z94009	Hydrolysis
867	*Beta proteobacteria*	*Burkholderiales/Comamonadaceae/Comamonas*	80	AB076865	acidogenesis
663	*Bacteroidetes*	*Unknown*	95	AY780554	unknown
1295		*Unknown*	87	DQ887931	unknown
983		*Bacteroidales/Rikenellaceae/Petrimonas*	93	AJ488088	acidogenesis/acetogenesis
565		*Bacteroidales/Rikenellaceae/Marinilabilia*	91	DQ141183	acidogenesis
569		*Bacteroidales/Porphyromonadaceae/Proteiniphilum*	78	EF559083	acetogenesis
274		*Bacteroidales/Porphyromonadaceae/Proteiniphilum*	68	AJ853498	acetogenesis
215		*Bacteroidales/Porphyromonadaceae/Proteiniphilum*	86	AJ853498	acetogenesis
175		*Bacteroidales/Porphyromonadaceae/Parabacteroides*	86	AB274509	Hydrolysis/ acidogenesis
113		*Bacteroidales/Porphyromonadaceae/Parabacteroides*	62	EF401205	Hydrolysis/acidogenesis
335		*Bacteroidales/Porphyromonadaceae/Paludibacter*	76	JX222159	propionic acid production
434		*Bacteroidales*	79	HQ602905	unknown
334		*Bacteroidales*	83	EF445286	unknown
1161		*Bacteroidales*	93	AY780552	unknown
750	Bacteria	*Unknown*	91	DQ887950	unknown
55		*Unknown*	76	AJ853533	unknown
518		*Unknown*	89	AJ853533	unknown
1384		*Unknown*	92	GQ479971	unknown

5.3.5.1. Dominant OTUs a low biogas methane content: A number of Clostridia OTUs, including the OTU 525 related to *Ethanoligenens* genus and the OTUs 1282, 114, and 214 related to *Ruminococcaceae* where dominant in digesters with low biogas methane content (Figure 5.8). This confirms as hypothesized in Chapter 3 that this family is associated with poor AD performance and high VFA concentration. *Ethanoligenens* are known to ferment a variety of mono-, di- and oligosaccharides with fermentation products including acetate, ethanol, hydrogen and carbon dioxide. In addition the nearest match to this OTU (DQ444185, Table 5.4) was a member of an anaerobic community degrading lactate, via sulphate reduction, to acetic acid, propionic acid or ethanol (depending on nutrient availability) (Zhao *et al.* 2008). This is significant as it indicates that the digesters have switched from syntrophic methanogenesis to syntrophic sulphate reduction pathways, which produce H_2S and CO_2 rather than methane and therefore undesirable as discussed in Chapter 4 (Van Den Berg *et al.* 1980; Gerardi 2003; Xing *et al.* 2006; Zhao *et al.* 2008). Two other dominant OTUs in digesters with a low biogas methane content were the *Parabacteroides* (OTUs 175 and 113) which were closely matched to two uncultured bacteria (AB274509) and (EF401205) isolated from an anaerobic packed-bed reactor degrading organic solid waste and the human gut respectively (Baena *et al.* 2000; Li *et al.* 2008).

The OTU 297 related to *Clostridium incertae sedis* XV was also dominant in digesters with lower biogas methane contents (Table 5.4 and Figure 5.8). In Chapters 3 and 4, OTUs related to *Clostridium incertae sedis* XV were dominant in digesters with low biogas methane content. The results here show that the OTUs 1271 and 1274 related to this family were also dominant in digesters with high biogas methane content. Also it was found in Chapters 3 and 4 that higher numbers of this family are associated with

faster recovery from high VFA concentration. Therefore *Clostridia* may be a good candidate group for bioaugmentation. Further to this all of the digesters during this study were able to return to optimal performance (in terms of biogas production). It should be investigated if pure cultures related to the OTUs dominant during periods of high VFA concentration are suitable for bioaugmentation to increase the degradation rate of VFA and improve digester recovery time.

5.3.5.2. Dominant OTUs at higher biogas methane content. Although the *Bacteroidales* order remained fairly constant in terms of total number sequences assigned to the order, the individual groups within the *Bacteroidales* varied largely. In particular there was a shift from the previously discussed *Parabacteroides* (OTUs, 175 and 113) to OTUs 983, 565, 596, and 274 as biogas methane content increased (Figure 5.8). OTU 983 was related to, the anaerobic fermentative *Petrimonas* which produces acetic acid, H+ and CO2 and therefore likely to play a role in syntrophic methanogenesis (Table 5.4). (Grabowski *et al.* 2005; Boone *et al.* 2010). The OTU 565 was related to *Marinilabilia* a producers of acetic acid, propionic acid, and lactic acid and therefore playing a role in acetogenesis and acidogenesis (Boone *et al.* 2010). The clusters 569 and 274 were related to *Proteiniphilum,* a genus known to be capable of degradation of propionic acid via syntrophic oxidation with methanogenic Archaea and hence an important group for maintaining stable AD (Chen and Dong 2005; Boone *et al.* 2010).

Overall digesters with low methane yields were associated with a higher number of fermentative *Ruminococcaceae,* including the genus *Ethanoligenens* which is related to sulphate reduction, this is consistent with the higher concentrations of VFA observed in

these digesters (Table 5.2). These findings indicate that during unstable period fermentative members dominate the bacterial community of the digesters with a possible switch to sulphate reductive pathways and reverse acetogenesis. In contrast when methane production was ≥ 46% there was a shift in the *Bacteroidales* members, especially a dominance of the *Proteiniphilum*, genus which is capable of syntrophic methane production in the presence of methanogenic Archaea. These results build upon the results presented in Chapter 3 confirming that variation in the specific OTUs identified are associated with AD performance.

5.3.6. Identifying important OTUs for AD performance optimisation

For optimising AD performance, it is important to understand if there are core groups of bacteria that are important for good performance (high biogas production, methane content, and stable production) and also to identify those that are associated with the worst performance. To do this the 6 previous digesters groups were further consolidated into 3 groups defined as low, medium and high biogas methane content (0-30, 31-60, and 61-85 %). The numbers of OTUs that were unique to each group and those that were shared are shown in Figure 5.9. Digesters with low methane content had the highest number of unique OTUs; in contrast the medium and high methane digesters had a lower number of unique sequences indicating that microbial group specialism occurred in anaerobic digesters with higher methane content. Syntrophic relationships between bacteria and methanogens are required for stable AD (Schnürer *et al.* 1999; Angenent *et al.* 2002; McMahon *et al.* 2004; Hattori. 2008; Stams and Plugge 2009), whereas in digesters with low methane production fermentation pathways, which are

fairly ubiquitous, dominate resulting in high bacterial diversity. Table 5.5 summarises the 15 most dominant unique OTUs for each digester group.

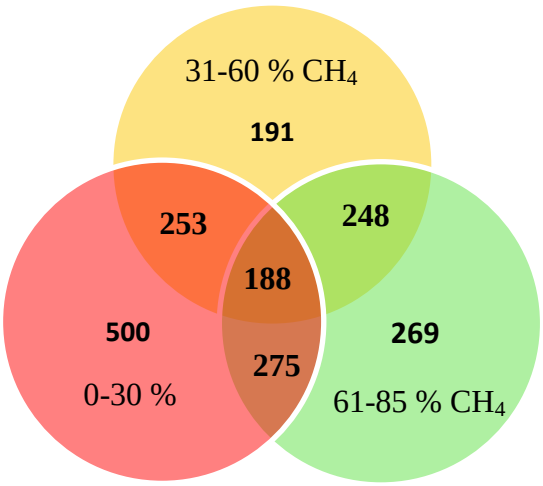

Figure 5.9. Venn Diagram showing number of unique and shared OTUs in digesters with 0-30 % (red), 31-60 % (yellow), and 61-85 % (green) biogas methane content.

The higher taxon distribution of the unique OTUs in each digester group is summarised in Figure 5.10, *Clostridia*les members were dominant in each digester group (> 45 %) which is unsurprising as they are known to play an important role in all bacterial stages of AD, hydrolysis (Schlüter *et al.* 2008; Kröber *et al.* 2009) and acido- and acetogenesis (Braun *et al.* 1981; Schwartz and Keller 1982; Karakashev *et al.* 2006). However they were particularly dominant in the low group at 62 %, supporting the hypothesis that increase in *Clostridia* is correlated with upset digester. This result is also significant as it shows that specific OTUs of *Clostridiales* are associated with each performance class, therefore monitoring for changes in *Clostridiales* OTUs structure as well as numbers can identify changes in performance of AD. The second most dominant order was the *Bacteroidales* in the high (9 %) and low (14 %) digesters groups, although they were

only 5 % in the medium digester group. *Burkholderiales*, *Acinobacteridae*, and *Acidomicrobidae* were also present at > 2 % in the medium and high groups.

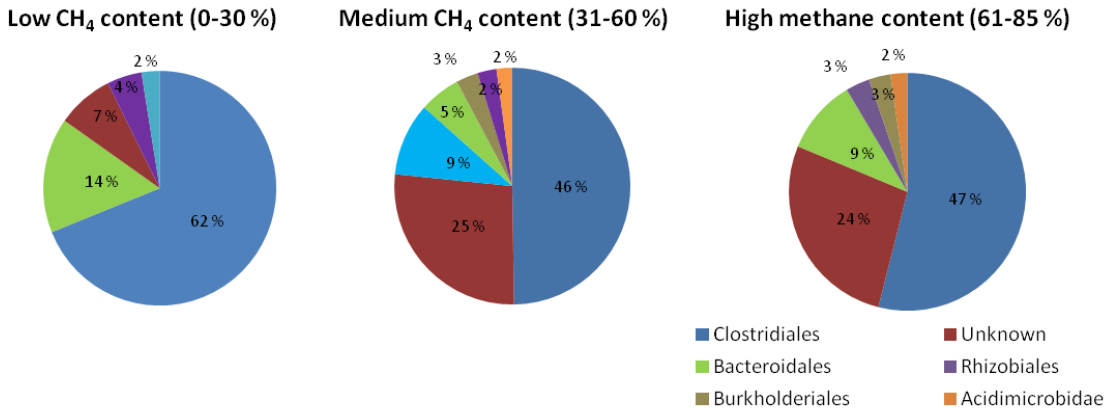

Figure 5.10. Higher taxon distribution of the unique OTUs in each digester group based on % OTUs abundance assigned at order level.

5.3.5.1. Unique Clostridiales: Analysis of the diversity within the *Clostridia*les (Figure 5.11) shows that as biogas methane content increases the number of unique *Ruminococcaceae* OTUs decreased twofold; this was accompanied by a threefold increase in unknown *Clostridiales* (Figure 8). This confirms than *Ruminococcaceae* are associated with poor methane production. In addition it suggests that the diversity of the *Clostridiales* associated with the high methane production is poorly defined as it was either unidentified or belonged to groups of uncertain placement "*incertae sedis*".

A number of unique dominant OTUs within *Clostridiales* in digesters with high methane content were related to syntrophic groups including the genus *Syntrophomonas*, *Cloacibacillus*, *Syntrophomonas*, and *Aminobacterium* (Table 5.5). The OTU 706, *Syntrophomonas* was most closely matched with the syntrophic fatty acid-beta-oxidizing bacteria *Syntrophomonas wolfei* (AF022248) which has been identified as playing a key role in preventing the accumulation of longer chain fatty

acids such as butyric acid (Hansen *et al.* 1999). Therefore it is possible to conclude that syntrophic *Clostridiales* play a key role in methane production via syntrophic methanogenesis. This finding further suggests that the feature of this bacterial group can be used for future AD optimisation strategy such as bioaugmentation. Indeed, Cavaleiro *et al.* (2010) demonstrated recently that anaerobic digesters bioaugmentated with *Syntrophomonas* had approximately 50 % reduced lag times and 30 % higher methane production from oleic acid.

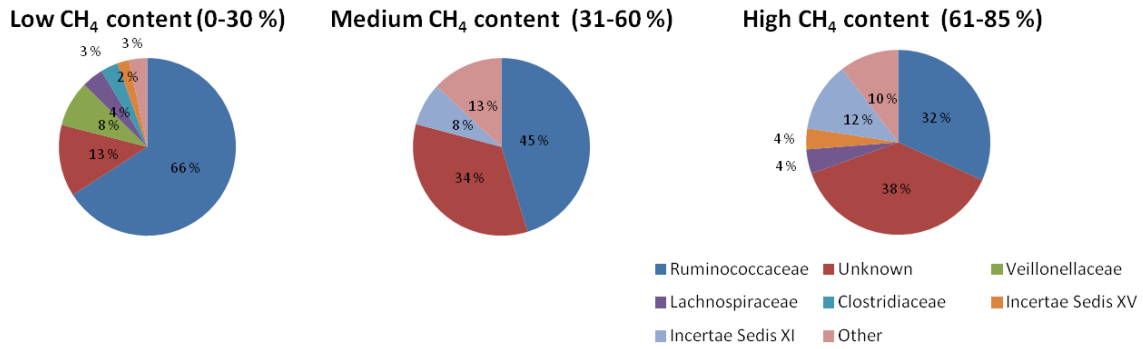

Figure 5.11. Distribution of the unique *Clostridia* OTUs in each digester group based on % OTUs abundance assigned at family level.

5.3.5.2. Unique Bacteroidales: 47 and 32 % of the unique Bacteroidales OTUs in the digesters with low biogas methane content are related to members of the Porphyromonadaceae and Prevotellaceae families, respectively (Figure 5.12). These family members were not detected in the medium and high methane digester groups. Specifically the OTUs 1205, 114, 1229, and 969 were dominant. The exact role of Prevotellaceae in AD is unknown but the closest matches to the representative sequences for the OTUs were both isolated from rumen and related to acidogenic bacteria (Whitford *et al.* 1998; Ramšak *et al.* 2000). Again there was a three-fold

increase in the numbers of unidentified OTUs, further highlighting the need for further research into the specific groups present in AD.

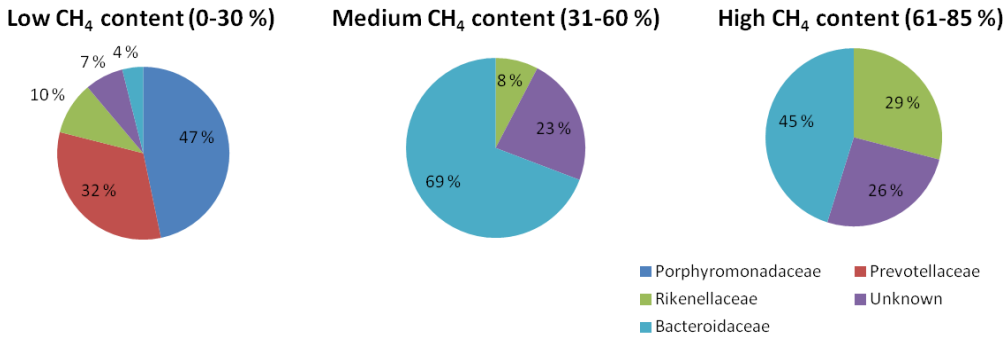

Figure 5.12. Distribution of the unique *Bacteroidales* OTUs in each digester group based on OTUs abundance assigned at family level.

Table 5.5. Summary of the 15 most dominant OTUs unique to digester groups.

Cluster	Higher Taxon	Class/Order/Family/Genus	Match %	Closest relative accession number	Probable function
Unique to low methane digesters group (0-30 %)					
1205	*Bacteroidetes*	*Bacteroidetes/Bacteroidales/Porphyromonadaceae/Parabacteroides*	78	EF515407	Hydrolysis/acidogenesis
1114		*Bacteroidetes/Bacteroidales/Porphyromonadaceae/Parabacteroides*	74	GQ458220	Hydrolysis/acidogenesis
1229		*Bacteroidetes/Bacteroidales/Prevotellaceae/Prevotella*	95	AF218617	acidogenesis
969		*Bacteroidetes/Bacteroidales/Prevotellaceae/Prevotella*	56	AF018486	acidogenesis
913		*Bacilli/Lactobacillales/Lactobacillaceae/Lactobacillus*	98	AF371488	acidogenesis
1147	*Firmicutes*	*Clostridia*/*Clostridia*les/*Ruminococcaceae*	70	AJ404681	acidogenesis
37		*Clostridia*/*Clostridia*les/*Ruminococcaceae*	82	GQ505048	acidogenesis
1341		*Clostridia*/*Clostridia*les/*Ruminococcaceae*	68	EU462528	acidogenesis
244		*Clostridia*/*Clostridia*les/*Ruminococcaceae* /*Oscillibacter*	74	EF402484	acidogenesis/long chain VFA
222		*Clostridia*/*Clostridia*les/*Ruminococcaceae* /*Acetivibrio*	70	JX224468	hydrolysis
108		*Clostridia*/ *Negativicutes/Selenomonadales/Veillonellaceae*	81	DQ223730	acidogenesis/long chain VFA
285		*Clostridia*/*Clostridia*les/*Clostridia*ceae	90	JN650228	Hydrolysis/acidogenesis
1268		*Clostridia*/*Clostridia*les/Veillonellaceae/Megasphaera	80	DQ223729	acidogenesis
988		*Clostridia*/Negativicutes/Selenomonadales/Acidaminococcaceae	67	AB509216	acdiogenesis
642	*Proteobacteria*	*Pseudomonadales/Pseudomonadaceae/Chryseomonas*	95	AY922098	acdiogenesis

Unique to medium methane digesters group (31-60 %)					
228	*Bacteria*	Unknown	57	AJ306784	
902	*Bacteria*	Unknown	60	GQ132440	
255	*Actinobacteria*	Actinobacteria/Actinobacteridae/Actinomycetales	54	EF688176	acidogenesis
1140		*Bacilli*/Bacillales/Planococcaceae/Planococcus	89	DQ298405	acidogenesis
166		*Clostridia*/*Clostridia*les	50	AB239487	Hydrolysis/acidogenesis
405		*Clostridia*/*Clostridia*les/*Ruminococcaceae*	69	AY330124	Hydrolysis/acidogenesis
456		*Synergistetes/Synergistia/Synergistales/Synergistaceae*	67	DQ478740	syntrophic/methanogenesis
605	*Firmicutes*	*Clostridia*/*Clostridia*les/*Ruminococcaceae*	64	JX225062	Hydrolysis/acidogenesis
721		*Clostridia*/*Clostridia*les/*Ruminococcaceae*	58	GQ134228	Hydrolysis/acidogenesis
997		Unknown	83	FN667366	
1313		*Clostridia*/*Clostridia*les/*Ruminococcaceae* /*Ruminococcaceae*	60	HQ697684	Hydrolysis/acidogenesis
301		*Clostridia*/*Clostridia*les/*Ruminococcaceae*	61	AB240338	Hydrolysis/acidogenesis
293	*Spirochaetes*	Spirochaetes/Spirochaetales	90	FJ461900	
132	*Proteobacteria*	*Gammaproteobacteria/Enterobacteriales/Enterobacteriaceae*	61	EU464477	acidogenesis

Unique to high methane digesters group (61- 80 %)					
289	*Acidobacteria*	*Acidobacteria*	43	HQ598419	acidogenasis
962	*Armatimonadetes*	*Armatimonadia/Armatimonadales/Armatimonadaceae/Chthonomonas*	52	AJ009490	hydrolysis
1463	*Chloroflexi*	Caldilineae/Caldilineales/Caldilineaceae/Caldilinea	73	EF632948	hydrolysis
182		*Bacilli*/Bacillales/Planococcaceae/Ureibacillus	60	GU320662	acidogenasis
216		*Clostridia*/*Clostridia*les/*Ruminococcaceae*	50	EF404556	Hydrolysis/acidogenesis
706		*Clostridia*/*Clostridia*les/Syntrophomonadaceae/Syntrophomonas	81	AF022248	syntrophic/methanogenesis
941		*Clostridia*/*Clostridia*les/Incertae Sedis XI	76	AF280819	
655	*Firmicutes*	Synergistetes/Synergistia/Synergistales/Synergistaceae/Cloacibacillus	57	AF280863	syntrophic/methanogenesis
399		*Clostridia*/*Clostridia*les	71	AY426448	Hydrolysis/acidogenasis
1473		*Clostridia*/ *Clostridia*les/*Ruminococcaceae*	53	EF559079	Hydrolysis/acidogenasis
1520		*Clostridia*/*Clostridia*les/Lachnospiraceae	62	EU843598	acidogenasis
24		*Clostridia*/*Clostridia*les/Syntrophomonadaceae/Syntrophomonas	61	JX542546	syntrophic/methanogenesis
25	*Synergistetes*	Synergistia/Synergistales/Synergistaceae/Aminobacterium	80	JX575852	acetogenasis/ hydrolysis (protein)
527	*Proteobacteria*	proteobacteria/Rhizobiales/Brucellaceae/Ochrobactrum	81	AY661464	nitrogen fixation

5.3.7. Correlation between bacterial community evenness and biogas methane content

Both community evenness and Shannon's diversity indices are higher in digesters with high biogas methane content (Table 5.6). A number of studies have reported that improved digester performance was related with higher community evenness (Wittebolle *et al.* 2009; Merlino *et al.* 2012; Werner *et al.* 2012). Similarly higher community evenness and bacterial production has been demonstrated in other environments such as bacterial communities on kelp fronds (Bengtsson *et al.* 2012). Linear regression was carried out to see if there was a significant correlation between community evenness and methane content or pH (Figure 5.13). Clearly a significant correlation between bacterial community evenness and methane content ($p = 0.007$, $R^2 = 0.5$) and bacterial community evenness and pH ($p = 0.01$, $R^2 = 0.48$) was observed for all conditions tested.

Table 5.6. Diversity indices of the digester groups

Digester group	**Low**	**Medium**	**High**
No. of unique OTUs identified (n)	500 (60 %)	191 (37 %)	269 (37 %)
No. of total OTUs identified	833	508	722
Community evenness (J')	0.48 ± 0.07	0.56 ± 0.09	0.64 ± 0.07
Shannon diversity index (*H'*)	1.3 ± 0.3	1.5 ± 0.1	1.7 ± 0.3

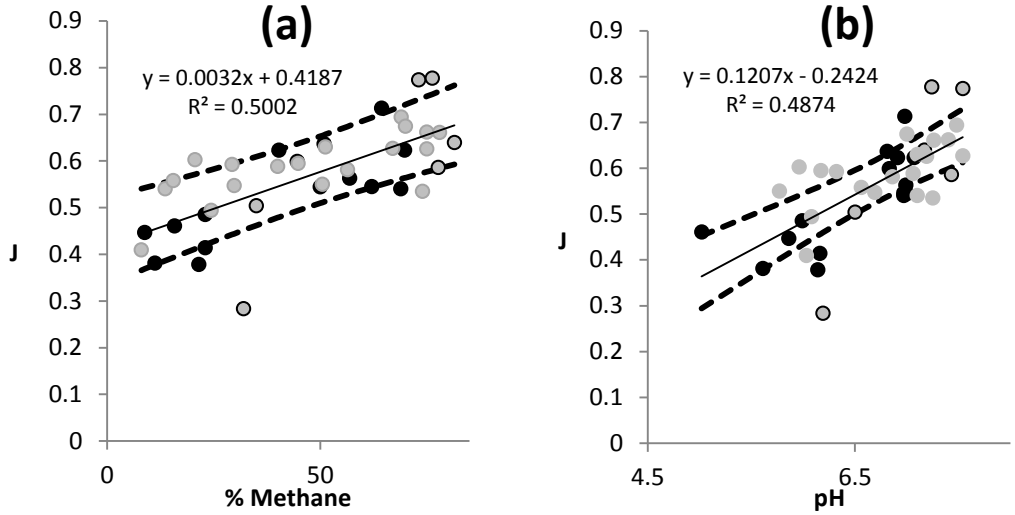

Figure 5.13. Scatter plot of Pielou's evenness index against % methane (a) and pH (b). (Solid lines represent linear regression between data points and dashed lines are 95 % confidence intervals of the predicted model). Pielou's community evenness index (*J'*) was calculated as described in section 5.27. Grey points with black border show co-digestion of FOGs with primary sludge, black points show glycerol co-digestion with primary sludge, and grey points with no border primary sludge only.

Table 5.7. Influence of the feedstocks on the relationships between community evenness, pH and percentage of methane

Feed	R^2		P	
	pH	% methane	pH	% methane
Primary sludge (PS)	0.36	0.49	0.006	<0.000
FOGs waste + PS	0.76	0.70	0.011	0.012
Glycerol waste + PS	0.56	0.62	0.001	< 0.001

In our study, evenness decrease was specifically related to low pH and poor methane production which can be related to microbial community stress, as stress will cause selection on the community resulting in the domination of stress resilient groups (Pesaro *et al.* 2004; He *et al.* 2012). Community evenness is particularly important in a system such as AD as high evenness enhances the opportunity to fully exploit all metabolic

pathways as well as the co-metabolic pathways which are known to play an important in AD performance (Hashsham *et al.* 2000; Werner *et al.* 2012). In this context, the increase of the fermentative *Clostridia* group (Figure 5.6) and the lower community evenness observed in the digesters may indicate imbalance in the functional stages of AD with an increase in production from the acidogenesis and acetogenesis stages. This imbalance is a known cause of digester instability as it results in pH decrease and changes in metabolic pathways (Van Den Berg *et al.* 1980; Conrad 1999; Schnürer *et al.* 1999; Zhang *et al.* 2007; Qu *et al.* 2009; Wang *et al.* 2009b; Laukenmann *et al.* 2011).

5.3.8. Archaeal community diversity and dynamics

Over 75 % of the archaeal diversity was dominated by the presence of members belonging to *Euryarchaeota* and especially to the genus *Methanosarcina* and to a less extent (only 1%) to the genus *Methanobrevibacter* (Figure 5.14). Further to this, 20 % of the OTUs identified were related to unidentified Archaea and a small number (< 2 %) of members belonging to *Thermoprotei*, a class of the *Crenarchaeota*,. Only three OTUs accounted for 54 % of all sequences, and no other OTUs had greater than 3 % of sequences. The closest matches to these OTU are summarised in Table 5.8. All are from the genus *Methanosarcina* which is the most metabolically diverse methanogenic genus and has also been shown to be the most stress resilient (Calli *et al.* 2005a; Calli *et al.* 2005b; Karakashev *et al.* 2005; Hori *et al.* 2006; Vavilin *et al.* 2008). It is therefore unsurprising that this group should dominate in all conditions analysed. These results highlight that diversity of the Archaea is low with only a small number of OTUs responsible for methane production in digesters across separate experiments with varying feedstocks. While there was no clear correlation between these groups and the

digester conditions, it is important to note that only 34 sequences on average were retrieved for each sample. A large number of the sequences retrieved were rejected due to short length. We are unsure as to the reason to this but preferential sequencing of short sequences or poor quality of the emulsion oil kit may have contributed to this outcome. However the sequences that passed the quality control were > 400 bp, therefore providing a good taxonomic resolution.

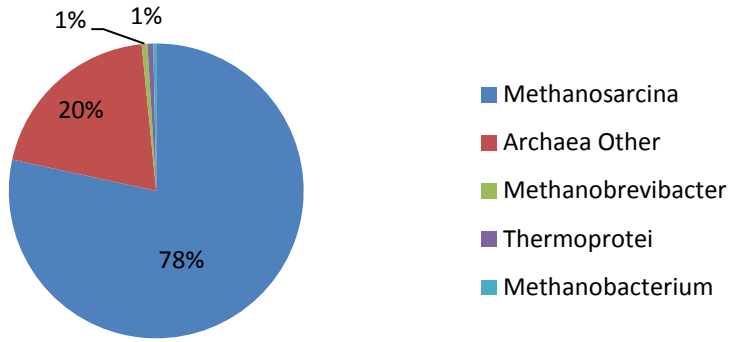

Figure 5.14. % Archaeal sequences assigned to genus or lowest possible taxonomic unit.

Table 5.8. Summary of dominant archaeal OTUs

OTU No.	% of sequences	Order/Family/Genus/Species	% Match	Closest relative accession number
1	**29**	*Methanosarcinales/Methanosarcinaceae /Methanosarcina lacustris* or *barkeri*	**83 %**	**AF432127 and AJ002476**
2	**16**	*Methanosarcinales/Methanosarcinaceae/ Methanosarcina*	**84 %**	**AF020341**

3	**10**	*Methanosarcinales/Methanosarcinaceae /Methanosarcinabarkeri*	**83 %**	**AF028692**

5.3.9. How can microbial community structure and dynamics information be used to monitor and optimise AD?

The microbial communities in AD are often treated as a black box and there is a general perception amongst AD operators that optimisation will not be achieved through an improved understanding the microbial ecology. In this study consistent shifts in the structure of the microbial communities have been observed with increase in biogas methane content. Such information can help to develop new strategies for monitoring and optimising AD process, and further assist AD operators to predict unstable digester performance.

5.3.9.1 Bioaugmentation: The core groups of bacteria specific to particular levels of performance revealed a number of unique OTUs in digesters with high biogas methane content related to *Syntrophomonas*, *Cloacibacillus*, *Syntrophomonas*, and *Aminobacterium* (Table 5.5). This information can further contribute to AD optimisation through monitoring or even via bioaugmentation. In Chapters 3 and 4, higher numbers of *Clostridium incertae sedis* XV and *Ruminococcaceae* were associated with faster recovery from periods of low biogas production in digesters with pre-experience of glycerol waste co-digestion. It was suggested that OTUs related to this group could also be used during periods of high VFA concentration to degrade VFA into acetic acid for methanogenesis.

5.3.9.2. Predicting performance: In Chapter 1 the potential to predict AD performance by monitoring the microbial community was discussed. Results presented in this chapter confirm this is possible. Analysis of the unique OTUs revealed a decrease in numbers of *Ruminococcaceae. Porphyromonadaceae* and *Prevotellaceae* with increasing biogas methane content Figure 5.11 and Figure 5.12. This was corroborated by the analysis of the dominant OTUs (Figure 5.8) and the higher taxonomic levels where *Clostridiales* dominated digesters with lower biogas methane content (Figure 5.6). The ratio of sequences assigned to *Clostridiales* and *Bacteroidales (*ratio C/B) was significantly higher (C/B = 2.7; p = 0.03) in the digesters with a methane content ranging between 0-30% than in the digesters with >60% methane (C/B = 1) Figure 5.15. This ratio may be detectable by culture-based techniques and further work is needed to see if this is possible. In addition to this the development of laboratory-on-chip systems will allow on-line monitoring of bioreactors as discussed in Chapter 1. These technologies could be used to monitor the relative proportions of *Clostridiales* and *Bacteroidales* based on sequence counts. They could also be used to detect variation at lower taxonomic levels, such as increases of *Ruminococcaceae* which were associated with poor performance, or increases in syntrophic OTUs (*Cloacibacillus*, and *Syntrophomonas*) which were correlated with the best performance. In this study *Clostridium incertae sedis* XV OTUs were observed at all levels of biogas methane content and therefore identified as a key fermentative group. Lipid analysis including PLFA and PLEL were also shown to add valuable insights to understand microbial communities structure and dynamics in digesters. A clear correlation between microbial biomass and methane production was observed. A combined approach of lipid fingerprinting and NGS provided detailed phylogenetic information with quantification of the microbial biomass thus delivering

further insights into AD communities. Further research is needed on these areas, but this study clearly demonstrates that microbial community structure and dynamics analysis can be used to monitor and optimise AD performance.

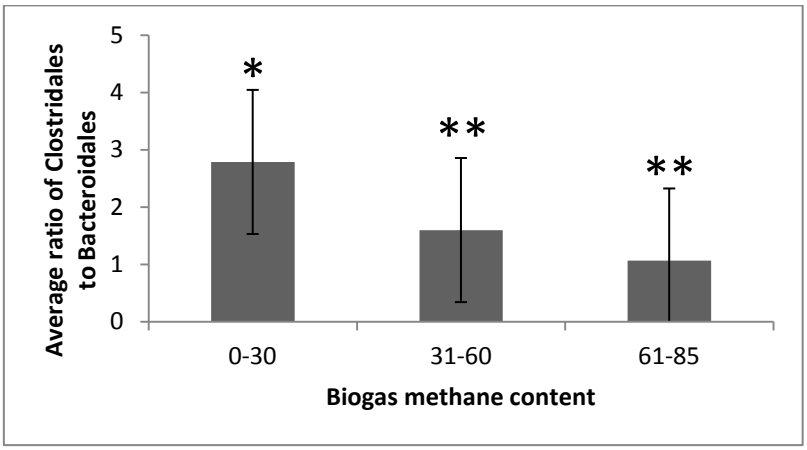

Figure 5.15. Average ratio of *Clostridales* to *Bacteroidales* in different digester groups based on biogas methane content. Error bars represent standard deviation and different number of * denote significant difference at p=0.05 using ANOVA in R (http://www.R-project.org/).

An updated version of the schematic linking operational parameters, with methane content and microbial community showed that there is a concomitant decrease in *Clostridia*, specifically *Ruminococcaceae* (Figure 5.16). Syntrophic bacterial groups were associated with high levels of methane biogas content and the *Bacteroidiales* genera and *Porphyromonadacrae* and *Prevotellaceae families,* were only found in digesters with low biogas methane content. The bacterial and archaeal biomasses based on the quantification of PLFA and PLEL respectively were positively correlated with biogas methane content.

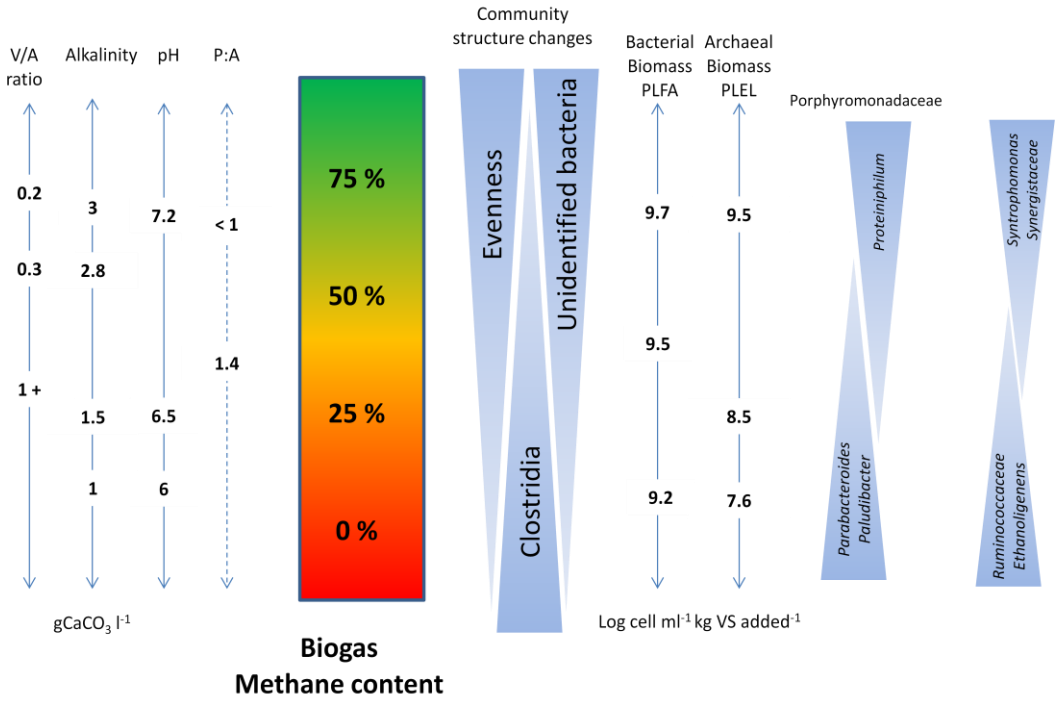

Figure 5.16. Summary of the key physicochemical parameters and microbial community features influencing digester performance**.**

5.4. Conclusions

The results clearly demonstrate a relationship between the community structure and the performance of AD. There were consistent increases in *Clostridia*, specifically *Ruminococcus* genus, in digesters with low biogas methane content. Further to this, a core group of unique OTUs related to optimal AD performance where identified. This included syntrophic bacterial groups related to *Syntrophomonas* genus family. A statistically significant correlation between community evenness and biogas methane content was also demonstrated, highlighting that a more equitable distribution of diversity in AD is related to higher methane production, possibly due to improved balance between the functional groups present.

It was also demonstrated that lipid fingerprinting, due to its ability to detect changes in biomass, is a valuable companion to sequence based analysis, or even on its own as a monitoring tool. Pyrosequencing analyses of multiple digester conditions in this study also revealed that a large proportion of sequences could not be assigned to taxonomic affiliations even at the phylum/class levels. This highlights that further work is required to fully understand the diversity present in AD.

Chapter 6: Key findings and Implications

6.1. Reminders of the PhD study aims

This PhD research aimed at improving methane production and process stability in AD by gaining a better understanding of the microbial ecology that drives the process. The influence of operational parameters, such changes in organic loading rate (OLR) and feedstock types on the microbial community response was investigated to identify the key microbial groups involved and determine the relationships between microbial community dynamics and digester performance.

It is well known that changes in feedstock and operational conditions influence the structure of the microbial community in AD. However little is known about how these changes in the microbial community structure affect the performance of digesters. As shown in Chapter 1, published results on the effects of performance parameters such as organic loading rate (OLR) on microbial community structure are inconsistent, and therefore presenting a contrasting picture for AD optimisation. Figure 6.1 (reproduced from Chapter 1 Figure 1.1) shows ways in which a microbial community in AD may respond to stress such as a change in OLR. Filling the gaps of knowledge highlighted in this figure will enable optimisation of the AD process.

To address this aim, five objectives related to the specific knowledge gaps in the field were defined and addressed in Chapters 2, 3, 4 and 5. The section 6.2 hereinafter summarises the key findings of each chapter.

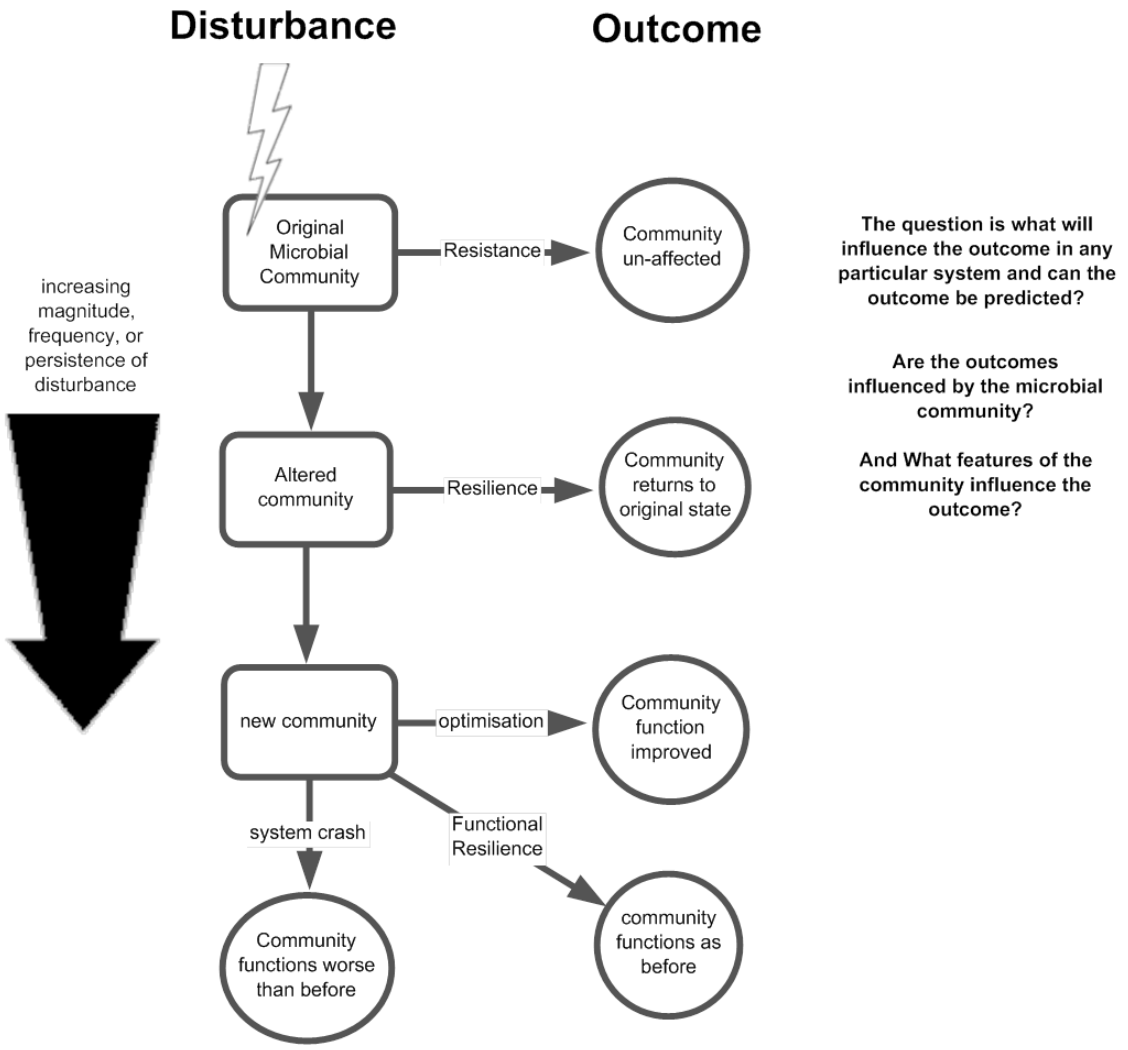

Figure 6.1. Theoretical response of a community to disturbance (adapted from Allison and Martiny 2008).

As discussed in Section 6.3 and Chapter 2 the performance of the microbial community was optimised to process high concentrations of VFA after prior exposure to OLR increase with glycerol waste. in terms of Figure 6.1, the original disturbance (glycerol waste OLR increase) resulted in an altered microbial community which showed optimisation when exposed to further glycerol waste OLR increase series (Figure 6.2).

When the new microbial community was exposed to FOGs waste OLR increase series it showed functional resilience and functioned as before. The key differences in function and bacterial and archaeal groups in terms of lipid markers and bacterial OTUs is summarised in Figure 6.3. Values for key operational parameters (Chapter 2) and microbial community structure (Chapters 3 and 5) in terms of methane production are summarised in Figure 6.4.

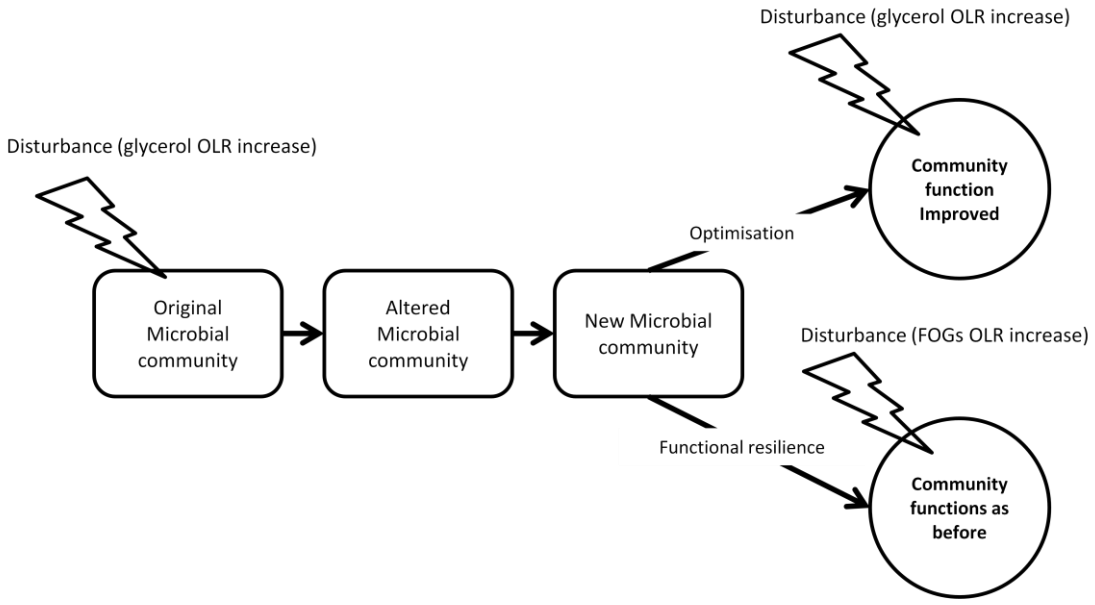

Figure 6.2. Summary of effect of OLR increase perturbation with different feedstocks in terms of Figure 6.1

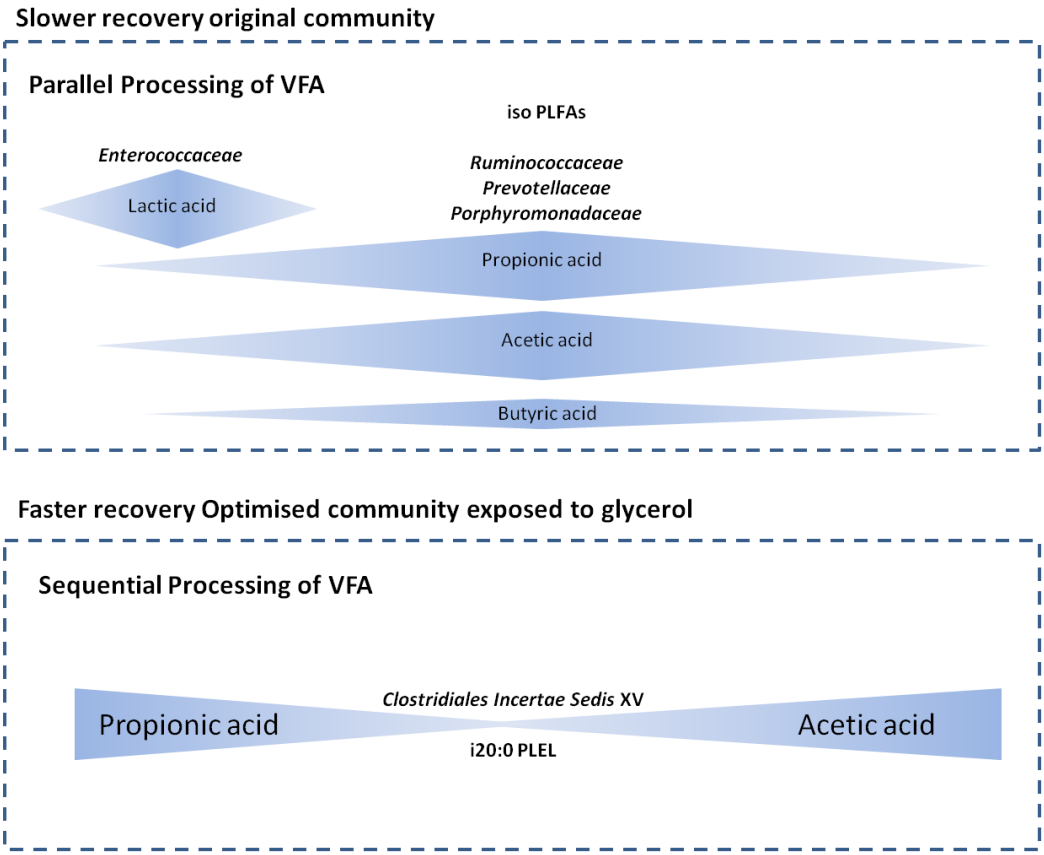

Figure 6.3. Key differences in microbial community (bacterial OTUs and lipid markers) and processing of VFA in the optimised and original microbial communities.

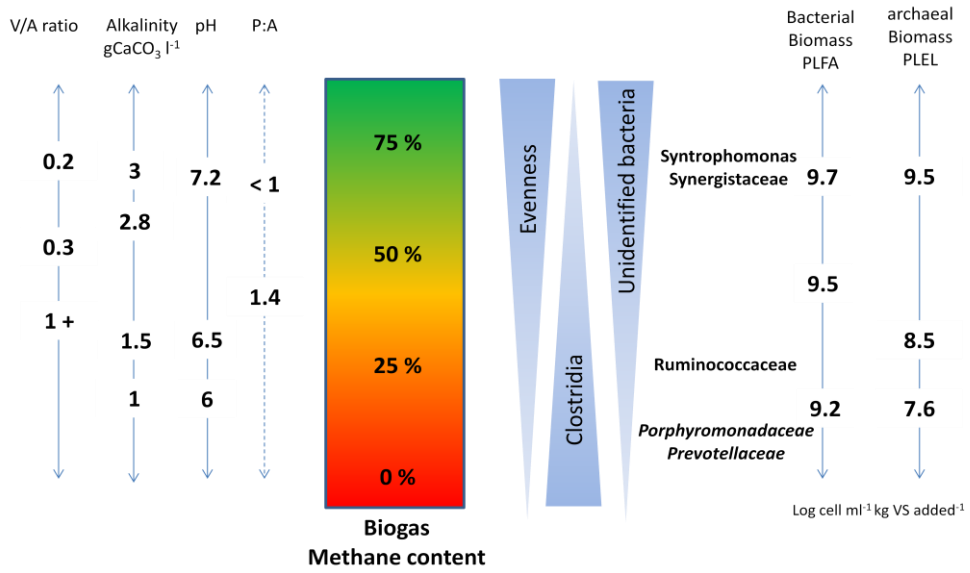

Figure 6.4. Summary of the key physicochemical parameters and microbial community features influencing digester performance.

6.2. Key results and implications

6.2.1. Effect of feedstock and OLR change on AD performance during co-digestion (Objective 2)

Chapter 2 aimed at understanding how the key physicochemical parameters in AD respond to single and multiple changes in OLR using glycerol waste and/or fat, oil and greases waste (FOGs waste) as co-substrate. Digesters were exposed either to a single OLR increase series with glycerol waste or FOGs waste as co-digestion substrates, or multiple OLR increase series using consistent feedstock composistion (glycerol waste) or varying feedstock composition (glycerol waste and FOGs waste). The effect of a higher OLR during OLR increase series was also investigated. Key process indicators including alkalinity, pH, propionic:acetic (P:A) ratio and V/A ratio to the different conditions were assessed to identify the most responsive parameter for monitoring process changes. The key novelty of this research was the use of multiple stress increase series and multiple feedstocks as many studies consider only single stress increase series or single feedstock type.

The results reported in Chapter 2 confirm that the response of digesters exposed to multiple changes in OLR varies depending on the past operation of the digesters. This demonstrates that short-term trials with single change in conditions such as batch tests do not provide a full picture of how a feedstock will perform in realistic operational conditions. This agrees with results published by McMahon *et al.* (2004) who also observed that digesters with history of unstable operation tolerated OLR increase better than those with a history of stable operation. This finding raises an important point for AD optimisation that long-term effects of changes in conditions need to be taken into account and that a failure can be advantageously used to optimise performance. The results also

demonstrate that although the relationship between V/A ratio and methane production rate is the same, irrespective of the past history of the digester or feedstock composition (V/A ratio increases when methane content in biogas decreases) the reference values can change depending on feedstock. V/A ratio vales were double for FOGs waste in comparison with glycerol waste, and ANCOVA revealed that the intercept of the linear regression between V/A ratio and biogas methane content was significantly lower ($p = 0.04$). Therefore the traditional reference values may need to be re-considered depending on the specific operational conditions of bioreactors. Propionic:Acetic ratio did not work as a consistent predictor of performance. However, in digesters exposed to both OLR increase series (digesters 4-6) the P:A ratio was far more dynamic than in digesters exposed to a single OLR increase series (digesters 1-3). This was related to differences in the way volatile fatty acids (VFA) accumulated, with VFA produced in parallel for the first increase series and in sequence for the second (propionic and then acetic acid). Changes in the way VFA are processed can result in improved AD performance and this has been linked to differences in microbial community structure (Fernández *et al.* 2000; Hashsham *et al.* 2000; Stroot *et al.* 2001; McMahon *et al.* 2004). It is concluded that P:A ratio alone is not sufficient to fully understand the AD process and that a more holistic understanding of all the VFA and intermediate products in AD and their links with the microbial community can improve long-term management of AD.

6.2.2. Effect of feedstock and OLR changes on microbial community during co-digestion (Objective 3)

Chapter 3 investigated whether the changes in digester performance could be related to changes in the structure and/or biomass of the microbial communities by using a combined approach of lipid fingerprinting and 454-pyrosequencing analyses. Specifically this

research provides new insights into the structure and abundance of the microbial communities in AD as follows:

- Digesters co-digesting glycerol waste had high proportion of *Clostridia Incertae Sedis* XV (38 %) and *Ruminococcaceae* (17 %).
- Digesters co-digesting FOGs waste (single increase series) had high numbers of candidate phylum OP10/ *Armatimonadetes* (26 %).
- OLR increase series resulted in a decrease of the archaeal biomass and an increase in *Clostridia* associated with a decrease of biogas production and methane content.
- These changes were also seen when digesters were exposed to multiple changes in OLR with glycerol waste and FOGs waste confirming that these parameters could form the basis of microbial monitoring indicators of AD.
- Higher numbers of *Clostridia incertae sedis* XV (closest cultivated match was *Synergistaceae Cloacibacillus* with 83% similarity) in the digesters exposed to multiple OLR increase series with glycerol waste suggest that they played a role in stabilizing digesters through syntrophic interactions with Archaea.

Further to this, Chapter 3 demonstrated that the improved recovery times observed in Chapter 2 were related to changes in the microbial response to OLR changes over multiple OLR increase series. This confirms that changes in operational conditions can be used to influence microbial community structure to optimise performance, as proposed by Briones and Raskin (2003). The shorter recovery times were linked to high numbers of *Clostridia incertae sedis* XV, suggesting that bioaugmentating digesters with this group can stimulate recovery after OLR increase. A statistically significant correlation between archaeal biomass and biogas methane content was found. This information could be useful for developing monitoring tools for AD process performance. Previous studies have pointed to

the suitability of lipid based fingerprinting for AD monitoring and this could be combined with high throughput PLFA for efficient monitoring of bacterial communities in AD (Buyer and Sasser 2012; Schwarzenauer and Illmer 2012). However at this stage nether the development of the technique or the understanding of what to monitor is sufficient to apply this to full scale monitoring of AD. The same is true of DNA based sequence monitoring of the AD community, as the high number of unknown bacterial groups is too much of a knowledge gap at present. Further analysis with high throughput PLFA techniques and sequencing technology are required to identify a core pool of bacteria and lipid markers that are related to stable/optimal AD. These could then be monitored via laboratory-on-chip systems which can either be used to monitor microbial communities based on physical characteristics (Morris *et al.* 2012) or DNA based methods such as DNA microarray (Talbot *et al.* 2008).

6.2.3. Effect of feedstock and OLR change on volatile fatty acids (VFA) production and microbial community dynamics in anaerobic digestion (Objectives 2 and 3)

In Chapter 2 faster recovery of digesters was related to changes in the way VFA were processed. In Chapter 4 a detailed analysis of the VFA profiles including lactic acid was carried out to further understand this. Multivariate analysis was also carried out to investigate the relationships between the VFA profiles, the lipid fingerprints and the bacterial OTUs identified in Chapter 3.

- Co-digestion of glycerol waste resulted in high production of lactic acid (5 ± 0.6 g l^{-1}) followed by parallel production of acetic and propionic acid (3.7 and 3.0 ± 0.6 g l^{-1} respectively)
- Co-digestion of FOGs waste generated two different stages of VFA production characterized by the accumulation of acetic acid (6.5 ± 0.6 g l^{-1}) and a high biogas

production followed by a period of accumulation of propionic acid (5.99 ± 1.01 g l^{-1}) and a poor biogas production.

- When digesters were exposed to repeated OLR increase series using glycerol waste, the production of VFA changed with no production of lactic acid. In contrast, there was no change in VFA profile when digesters were exposed to repeated increase in OLR using different co-digestion substrates (glycerol waste and FOGs waste).
- Multivariate analysis demonstrated that the production of lactic, propionic and iso-butyric acids was correlated with the high numbers of *Rumminococcaceae, Entrococcaceae, Porphymonadaceae, Prevotellaceae.* Further to this, the concentrations of the bacterial lipid markers iso17:1, iso15:0 and iso16:0 were correlated to the production of lactic, propionic and iso-butyric acids.
- The bacterial members belonging to *Clostridia incertae sedis* XV and the concentration of the archaeal lipid marker i20:0 were correlated to the concentration of acetic acid.

The results confirm as proposed in Chapter 3 that high numbers of *Clostridia incertae sedis* XV/*Synergistaceae* and higher methanogenic biomass uphold the conversion of lactic, propionic and n-butyric acids into acetic acid and ultimately methane via syntrophic relationships with archaea. This research further highlights the importance of the syntrophic relationships between bacteria and archaea in AD as highlighted by other authors (Sekiguchi *et al.* 2000; McMahon *et al.* 2004; Hori *et al.* 2006; Hattori. 2008; Schauer-Gimenez *et al.* 2010; Chen *et al.* 2012; Westerholm *et al.* 2012). It is clearly important that strategies to enhance and strengthen these relationships in AD are found as they can lead to process optimisation. Past stress such as OLR increase, high ammonia or

VFA concentration can increase numbers of syntrophic archaea and bacteria (Griffin *et al.* 1998; McMahon *et al.* 2004; Calli *et al.* 2005a; Calli *et al.* 2005b; Karakashev *et al.* 2005; Karakashev *et al.* 2006; Banks *et al.* 2012; Chen *et al.* 2012; Ros *et al.* 2013) and syntrophic groups can also be directly bioaugmented into digesters (Cavaleiro *et al.* 2010; Schauer-Gimenez *et al.* 2010; Tale *et al.* 2011). Both should be developed for AD optimisation.

The relationship between archaeal community structure and VFA is well understood (Griffin *et al.* 1998; Delbès *et al.* 2001; Karakashev *et al.* 2005; Hori *et al.* 2006; Ros *et al.* 2013). However, our understanding of the relationships between the bacterial community dynamics and the VFA composition and concentration is still limited. The results reported in Chapter 4 contributed to shed light on this aspect. Information about how bacterial groups are related to VFA production is important for AD optimisation as changes in bacterial dynamics are more relevant to AD optimisation when they are related to a change in AD performance. The number of members belonging to the bacterial families *Rumminococcaceae*, *Entrococcaceae*, *Porphymonadaceae* and, *Prevotellaceae* and the iso lipid markers (iso17:1, iso15:0 and iso16:0) concentrations were related to the high concentration of > 2 carbon VFA. Therefore these bacterial groups may be associated with poor performance and could be suitable for monitoring. Not all changes in bacterial community structure could be related to changes in AD function in this research. Microbial communities in digesters exposed to either a single OLR change using glycerol waste or FOGs waste or multiple changes of OLR using both glycerol waste and FOGs waste were shown to be distinct in Chapter 3. The analysis of the VFA profiles did not associate this with any change in metabolic pathways being carried out by the bacteria. This demonstrates high functional redundancy in these microbial communities. This feature

could represent a barrier to microbial optimisation of AD because it complicates the identification of core groups of bacteria that can be related to performance in AD (Fernández *et al.* 1999; Fernández *et al.* 2000; Zumstein *et al.* 2000; Wang *et al.* 2010b; Wang *et al.* 2011). However, the use of combined techniques such as lipid profiling and high throughput sequencing, as used in this research, can provide the resolution required to establish the relationships between the bacterial and archaeal groups function with the process performance. In particular it is important to understand shifts in bacterial groups from a more functional perspective if insights into process optimisation are sought after.

6.2.4. Microbial community dynamics and anaerobic digester performance (Objectives 4 and 5)

Results in Chapter 3 suggest that there was a relationship between the microbial community structure and the digesters performance. Chapter 5 provides further insights with the aim of determining whether there are consistent relationships between microbial community structure or biomass and AD performance. To ease the investigation, the samples were split into groups based on the biogas methane content. Key findings are as follows:

- A 50% increase of the sequences from *Firmicutes* (particularly in OTUs related to *Ruminococcus*) was correlated with a decrease in biogas methane content to less than 30 %.
- Increase in biogas methane content to > 60 % was associated with increasing numbers of unidentified OTUs.
- A more even distribution of diversity was correlated with increased biogas methane content.
- Archaeal diversity was dominated by the genus *Methanosarcina*

The lack of reliable monitoring tools has been highlighted as a barrier to fully optimise AD process (Ward *et al.* 2008). Furthermore the value of understanding the biological component of waste treatments systems is often underestimated as AD optimisation is often carried out using chemical engineering options (Briones and Raskin 2003; Graham and Smith 2004; McMahon *et al.* 2007; Wang *et al.* 2011; Valentin-Vargas *et al.* 2012). In Chapter 5 consistent shifts in the structure of the microbial communities have been observed with decrease in biogas methane content (increase in *Ruminococcus*) providing proof of concept that such information can help to develop new strategy for monitoring and optimising AD process, and assist AD operators to predict unstable digester performance. However a high number of sequences related with high biogas methane content could not be assigned to taxonomic affiliations, highlighting that further work is required to fully understand the diversity present in AD. Communities with a more even distribution of diversity were correlated with higher biogas methane content. It is well known that imbalance in the functional stages of AD can result in accumulation of VFA, pH decrease, and reduced methane production (Zoetemeyer *et al.* 1982; Appels *et al.* 2008). This was consistent with our results as less even communities were associated with low pH (< 6.5) and higher concentrations of VFA. It can be speculated that less even communities (which were dominated by fermentative *Clostridia* such as *Ruminococcus*) were imbalanced between the VFA producing and methane producing stages resulting in accumulation of VFA. Research with a more functional based approach to identify numbers or activity at the various microbial stages of AD would be required to confirm this. However, the fact that biogas methane content was high (61-85 %) when the ratio of *Clostridales/Bacteroidales* was 1:1 and the biogas methane was low when the C:B ratio was 3:1 further evidenced that even distribution of functional groups in AD is important. If

specific functions could be assigned to the bacteria in AD (the function of the archaeal competent is well known) then it would be possible to quantify changes in the abundance of the functional stages of AD (hydrolysis, acidogenesis, acetogenesis) in the context of AD performance. If functional redundancy is as significant as suggested by some previous research, with functional traits spread across a high diversity of bacteria, then a functional based analysis of the bacterial community may be the best way of developing monitoring tools for AD.

6.3. Recommendations for AD operators

6.3.1. Glycerol waste and FOGs waste as substrates for co-digestion

A number of different feedstock compositions were used in this study, although the aim was to cause process failure by overloading the digesters, some recommendations for co-digestion of glycerol waste and FOGs waste can be made.

Table 6.1. Biogas production, methane content, and optimal concentration for different feedstocks.

	Units	Primary sludge	Primary sludge +	
			Glycerol	FOGs
Best biogas production	m^3 Kg VS day^{-1}	0.18	0.14	0.25
Average % methane	%	70 ± 9	53 ± 5	79 ± 8
optimal concentration	g l^{-1} glycerol /FOGs	NA	0.75	0.9

Addition of glycerol waste: Addition of glycerol waste at 0.75 g l^{-1} VS glycerol waste day $^{-1}$ was the maximum concentration that was able to sustain stable methane production (Table 6.1). However even at 0.75 g l^{-1} glycerol waste day $^{-1}$ the % methane in biogas decreased from ≈ 70 % (primary sludge) to 53 % indicating that the fermentation of glycerol waste resulted in the production of CO_2 and did not provide any significant increase in methane production. Addition of glycerol waste at any higher concentration than 0.75 g l^{-1} VS glycerol waste day $^{-1}$ resulted in severe inhibition of methane production, the total biogas production decreased from 0.14 m^3 Kg VS day $^{-1}$ to < 0.01 m^3 Kg VS day $^{-1}$ and the biogas methane content decreased from 69 % to 20 %. Other studies have reported similar to results, with OLR > 2.10 g VS l^{-1} (corresponding to 1 g l^{-1} VS glycerol waste day $^{-1}$) resulting in accumulation of VFA and process failure (Martin *et al.* 2013). Conversion of glycerol waste into methane may not be the best use for glycerol waste. Glycerol waste can be biologically converted into a number of other high value products including 1,3-propanediol, butanol, ethanol, and formate (Yazdani and Gonzalez 2007). In this study, high concentrations of lactic acid were also achieved (Chapter 4). Further to this, there was evidence that co-glycerol waste digestion lead to sulphate reduction. Therefore a more viable use for waste glycerol waste could be as a carbon source for sulphate reducing bacteria for biological removal of sulphate from wastewaters as shown by Dinkel *et al.* (2010).

Addition of FOGs: The best production of methane was achieved at a concentration of 0.9 g l^{-1} FOGs in feed (corresponding to a OLR of 3.2 g l^{-1} VS day $^{-1}$) which resulted in 0.25 m^3 Kg^{-1} VS added which is significantly higher than the production of biogas for primary

sludge alone or glycerol waste co-digested with primary sludge (Table 6.1). In addition to this, methane content of > 90% was recorded in digesters fed with primary sludge supplemented with FOGs which is the highest for any feedstock combination. At concentrations higher than 1 g l^{-1} FOGs in feed, methane production was inhibited. This is in good agreement with Palatsi *et al.* (2009) who also reported inhibition at FOGs waste concentration higher than 1 g l^{-1} FOGs in feed. However the high methane yields resulting from co-digestion of FOGs make it an attractive strategy for treatment of lipid rich waste streams such as FOGs if inhibition can be pr increase series ed. This is not new and many studies have investigated strategies for AD of LCFA (Cirne *et al.* 2006; Cirne *et al.* 2007; Hatamoto *et al.* 2007; Sousa *et al.* 2007; Sousa *et al.* 2008; Alves *et al.* 2009; Long *et al.* 2011; Martin-Gonzalez *et al.* 2011). The results in this study showed that after recovery from inhibition caused by overloading of FOGs the performance in terms of biogas production was reduced by 42 %. This must be considered by AD operators as it suggests that the consequences of process inhibition by FOGs overloading are long-term. The results reported in this research showed that prior exposure to stress caused by an increase in OLR using glycerol waste did not result in improvement in term of performance when exposed to a second OLR increase when FOGs was used. Therefore other strategies for recovering digesters from FOGs overload are needed. Palatsi *et al.* (2009) showed that the best options may include increasing the biomass/FOGs ratio, addition of adsorbents for adsorbing the FOGs, reducing the bioavailable FOGs concentration, and acclimation of biomass to FOGs.

6.3.2. Establish operational parameters

Results in Chapter 2 showed that operational monitoring parameters such as V/A ratio and P:A were affected by changes in feedstock. Bacterial biomass was also lower in the digester set 2 in Chapter 5. This suggests that values for operational parameters may vary for specific digesters and therefore AD operators may have to establish the precise operational values for their own specific systems.

6.4. Wider implications of findings

The research presented is of wider interest to microbial ecologists and community ecologists in general. In particular the positive relationship between evenness and biogas methane content which builds upon previous research relating community structure to function and stability but at a higher level of biodiversity (Bell *et al.* 2005; Girvan *et al.* 2005; Wittebolle *et al.* 2009). AD systems represent a good model system for answering fundamental questions about the relationships between community structure and stability and/or function (Jessup *et al.* 2004). Small microcosm communities comprising 10-100 species have been used to investigate the relationship between biodiversity and ecosystem functioning in the laboratory (Bell *et al.* 2005; Wittebolle *et al.* 2009; Gravel *et al.* 2010). However it is not known if this relationship persists in more complex microbial communities which have much higher biodiversity. Experiments in bioreactors such as those used in AD studies may represent a suitable way to investigate these effects in more realistic natural communities. The information learnt about how biodiversity effects ecosystem functioning and stability will feedback into better understanding of how to engineer and operate AD systems to further optimise the AD microbial community (Briones and Raskin 2003; McMahon *et al.* 2007; Harmand *et al.* 2008).

6.5. Limitations

6.5.1. Measurement of biogas

Biogas production was measured by displacement of acidified water (pH = 2). Studies have shown that although this results in less error than tap water that there is still significant loss of carbon dioxide and methane to gas absorption (Muller *et al.* 2004; Walker *et al.* 2009). Acidified saturated NaCl solution has been suggested as a more appropriate barrier solution for measuring biogas in AD due to lower solubility of gases in this solution. Regular measurement of biogas production (six days a week) was employed to reduce the time available for biogas to be lost by absorption. Further to this, samples for gas composition were collected from the headspace of the digester and measured immediately to pr increase series error from differential absorption of methane and carbon dioxide in the solution. Other methods for measuring biogas production such as gas flow meters, could have been used but are costly. It is therefore recommended for future work that saturated NaCl solution should be used to minimise loss of methane, as this represent no increase in cost but a significant increase in accuracy.

6.5.2. Scale of digesters

All of the studies carried out in this thesis where carried out either in 1 or 5 L scale. The application of results on microbial communities in lab scale studies has been criticised by Valentin-Vargas *et al.* (2012). The use of lab-scale digesters was unavoidable to allow replication of results in triplicate. Replication was required to take into account for differences microbial communities between individual digesters due to niche specific factors, which have been shown to be significant in AD systems (Werner *et al.* 2012).

However caution in extrapolating lab-scale findings should be considered when applying to full-scale systems, as a full-scale AD plants will provide a wider range of conditions/niches for microbial groups which may fundamentally change the diversity and dynamics of the microbial community. Indeed neutral theories of biogeography suggest that larger reactors will have higher microbial diversity (Curtis and Sloan 2004). However microbial groups such as methanogens which have a low functional redundancy and diversity have been found to be more replicable across special and temporal scales than bacteria in AD (Godon *et al.* 1997; Fernández *et al.* 1999; Delbès *et al.* 2000; Zumstein *et al.* 2000; Delbès *et al.* 2001; Curtis and Sloan 2004; Leclerc *et al.* 2004). This can also be applied to the more specialist groups of bacteria which also show less functional redundancy and diversity. This has been demonstrated by Werner *et al.* (2012) who found that syntrophic and specialist bacteria could be related to functional conditions in AD. It is therefore plausible that relationships between microbial communities structure found at lab-scale still apply to full-scale systems when looking at specialist microbial groups with lower functional diversity. These include members belonging to *Synergistaceae* family which were related to good performance in this study (Chapters 3 and 5).

6.5.3. Inferring function of microbial groups

This study used 16S rDNA analysis to understand the taxonomic diversity of bacteria and Archaea present in the digesters. Relationship between the changes in taxonomic diversity and the function of the digesters was then investigated using multivariate analysis. However, as observed in this study vastly different bacterial communities were present when digesting FOGs despite similar performance. Many other studies have observed high functional redundancy in bacterial communities in AD (Fernández *et al.* 1999; Wang *et al.*

2010b; Feng *et al.* 2011; Wang *et al.* 2011). Therefore it may not always be appropriate to infer changes in function with changes in community structure. A metagenomic approach analysing the microbial community based on functional role would be appropriate for further study.

6.6. Recommendations for further research

6.6.1. Modelling AD

Models of the AD process such as IWA Anaerobic Digestion model No.1 (ADM 1) developed by Batstone *et al.* (2002) only considered the different microbial groups involved in the functional stages of AD process and do not consider diversity between organisms with the same function (Ramirez *et al.* 2009; Appels *et al.* 2011). The works of Ramirez and Steyer (2008) and Ramirez *et al.* (2009) represent the first attempt to account for diversity between organisms with the same function in AD and resulted in an improvement of the ADM 1 model, but the application of NGS data to understand function of specific groups in AD is necessary to further improve modelling of AD.

6.6.2. Long-term trials

A long-term analysis (600+ days) of the microbial community in an bioreactor showed that an extremely dynamic community can maintain a stable ecosystem function (Fernández *et al.* 1999). The results in this thesis further showed that the function of the community is influenced by past conditions. Despite this, many trials rely upon single sampling increase series and do not provide a comprehensive insight into long-term function in AD (Leclerc *et al.* 2004; Schlüter *et al.* 2008; Kröber *et al.* 2009). The use of multiple sampling days

from a variety of different biogas plants with varying conditions are required to understand the long-term functioning of AD.

6.6.3. Biodiversity ecosystem functioning studies

Artificiality manipulated bacterial communities have been used to answer fundamental ecological questions about how community structure relates to ecosystem function, information which in turn could be used to optimise AD communities (Bell *et al.* 2005; Gravel *et al.* 2011). Much of the research in this regard has focused on artificially created and manipulated bacterial communities of < 100 species and the results may not scale to the complex natural communities. As discussed in section 6.4 AD bioreactors are good model systems for investigating fundamental questions about ecology and the research outcomes can feedback into AD optimisation directly (Briones and Raskin 2003; Jessup *et al.* 2004; McMahon *et al.* 2007). Studies have been carried out to understand how community respond to different conditions (resilience, adaptation, speciation) in AD (Delbès *et al.* 2000; Fernández *et al.* 2000; Hashsham *et al.* 2000; Stroot *et al.* 2001; McMahon *et al.* 2004). Many studies only compare a small number of conditions, often only 2, a wider range of diversities would provide a greater insight via truly correlative analysis such as shown by Bell *et al.* (2005). A way of achieving this would be to serially dilute a seed culture to produce communities with decreasing diversity as done by Wertz *et al.* (2006) in a study on the influence of soil microbial diversity on soil ecosystem processes. This approach may provide new insights into the effect of biodiversity on ecosystem function in AD.

6.6.4. Need to understand the rare and unidentified diversity

A high proportion of the OTUs identified, particularly in digesters with high methane production, were unable to be identified to phylogenetic level greater than domain (> 45 %, Chapter 5). Furthermore, OTUs were detected from groups such as TM7 that have only been identified from environmental 16S rRNA sequences (never isolated or cultured) (Table 6.2). This is consistent with the literature (Chouari *et al.* 2005a; Chouari *et al.* 2005b). It is important that research is targeted at better understanding these groups as they could contribute to optimise AD. Examples of how this can be done include research carried out by Chouari *et al.* (2005a) which specifically targeted the less understood groups in AD and provided new insights into these groups.

Table 6.2. Summary of OTUs of uncertain placement identified in this study.

Taxonomic group	Number of OTUs detected
OP10/*Armatimonadetes*	15
Clostridia incertae sedis III	1
Clostridia incertae sedis IV	3
Clostridia incertae sedis XI	50
Clostridia incertae sedis XII	1
Clostridia incertae sedis XIII	13
Clostridia incertae sedis XV	53
TM7	4

6.6.5. Understanding the function of the bacterial groups

As discussed previously, bacterial communities with a more even distribution of diversity produced higher biogas production. They may also have greater stability (Wittebolle *et al.*

2009). It was speculated that less even communities were imbalanced between the functional stages of AD resulting in poor performance. To confirm this, research would require concentrating efforts on the function rather than identity of the bacteria and archaea present in AD. A metagenomic approach can assist in this task as shown in studies of bacteria community associated with green macroalga *Ulva australis* (Burke *et al.* 2011) or in AD by Abram *et al.* (2011) and Kröber *et al.* (2009).

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Appendix A - Initial experiments carried out with glycerol and FOGs co-digestion.

Preliminary glycerol work

Previous work at Cranfield University

Initial work with glycerol co-digestion was carried out by a masters student before the PhD. "Alitimira F. Co-Digestion of sewage and glycerol: Monitoring of methane production and volatile fatty acids. In: School of Applied Sciences, Cranfield University (2008)". Glycerol was co-digested in batch at 1 % and 3 % of feed with sewage sludge. Digesters with 1 % glycerol had the best performance with a 10 % improvement in biogas methane content and a threefold increase in biogas production to 13.34 cm^3/gVS. Digesters with 5 % glycerol did not perform well with biogas methane content of 14 %, total biogas production however was higher than in all other treatments at 19.92 cm^3/gVS. This reflects high production of CO_2 during glycerol co-digestion. This work set the initial parameters for glycerol co-digestion in this PhD thesis. As the previous work was in batch further work was required to optimise the system for continuous operation and to asses if digesters could recover from glycerol concentrations of 3 % and greater in feed.

Preliminary trials with glycerol

Glycerol was used as co-substrate to stress the community at 1, 3, and 5 % of feed which corresponds to 10, 30 and 50 gl^{-1} glycerol in feed. The headspace of the reactors was flushed with nitrogen prior to the start of the experiment. Gas production and methane content was measured daily, pH was measured every two days by analysis of the reactor effluent.

Aims of this study were to;

- Determine suitable operational conditions, such as hydraulic retention time for continuous operation of the anaerobic digester rig.
- Determine if the system could recover from inhibition caused by glycerol.
- Determine if the performance would change over multiple additions and recovery from glycerol
- Investigate the possibility of loading glycerol at a higher rate with a semi-continuous system

Conclusions

- At an HRT of 7 days is was possible to achieve > 50 % volatile solids reduction. With longer HRT biogas production decreased indicating fresh feed was required to prevent starvation.
- Glycerol addition of 0.5% does not have any effect on stability but decreases biogas methane content from 70 % to 50 %.
- The reactors fed with 3 % and 5 % glycerol where able to return to stable operation within 10 days of glycerol addition.
- There was no evidence of system acclimatisation over multiple glycerol loadings.

Problems with this work

The digesters were fed with material from the anaerobic digesters at a local sewage treatment works. This feed was unsuitable for investigating the influence of multiple additions of glycerol as it allowed re-seeding of digesters in-between shock events. In further work an autoclaved feed was used to avoid this.

Preliminary work with FOGs

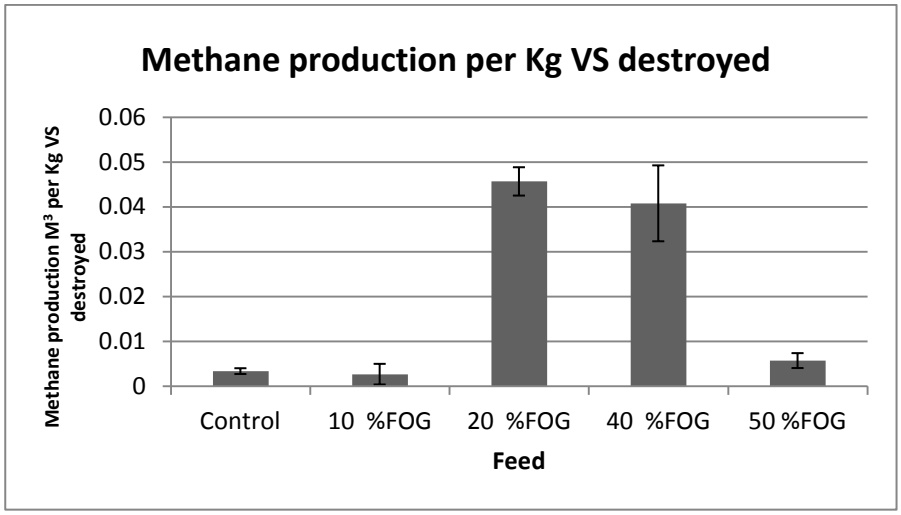
FOGs were collected from a grease trap at a restaurant on campus. The FOGs collected were diluted with water to achieve a consistent concentration for use as feed.

Digestibility batch trial

Triplicate reactors were fed with a mixture of primary sludge and FOGs as shown in table. The headspace of the reactors was flushed with nitrogen prior to the start of the experiment. Gas production and methane content was measured daily.

Primary sludge (%)	FOGs waste (%)	Concentration of FOGs (gl^{-1})
100	0	0
90	10	0.5
80	20	1
60	40	0.8
50	50	2

Results showed inhibition of AD at concentrations of 2 gl^{-1} FOGs in feed. At lower concentrations the biogas methane production was significantly increased. > 50 % VS reduction was achieved and no significant biogas production was observed after 7 days, confirming that 7 days was suitable for HRT.



Inhibition trial

A trial was carried out to determine at what concentration of FOGs in feed AD was inhibited and if digesters could recover. Duplicate reactors (ten total) were seeded with a 20:80 mix of AD digestate and autoclaved primary sludge supplemented with FOG at 0.90, 3.20, 6.40 and 8.00 gl^{-1}. The headspace of the reactors was flushed with nitrogen prior to the start of the experiment. Gas production and methane content was measured daily, pH was measured every two days by analysis of the reactor effluent. 4ml of 1moler NaOH was added to reactors with a pH of less than 7.00 every day to correct pH.

Results

Results showed that recovery from FOGs co-digestion was not achievable even after 60 days at concentrations higher than 3.2 gl^{-1} FOGs in feed, even with pH correction. Therefore a concentration 1.5 gl^{-1} FOGs waste in feed was suitable for causing digester upset with a realistic chance of recovery.